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SECTION E

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STATISTICAL METHODS IN MEDICAL RESEARCH

I. QUALITATIVE STATISTICS (ENUMERATION DATA)[1]

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Abstract

This article is designed to help investigators in applying to qualitatively classified clinical and laboratory data the appropriate statistical treatment—tests of significance in binomial and multinomial distributions, estimation of confidence limits, analysis of contingency tables, and estimation of sample sizes required for further investigation. Section A is a brief introduction (definitions and principles). Section B comprises 40 examples classified so that the investigator can choose data and problems comparable to his own. Questions that arise in the examples, regarding experimental design (especially random sampling) and the interpretation of the tests, are discussed in Section C (Notes).

Because the standard deviation of the binomial and the chi square contingency test are often used without appreciation of the risk entailed, tables, which can be used also in nonmedical investigation, are presented: binomial confidence limits (with graphs) and exact probabilities for small-sample fourfold contingency tables. For samples not covered by the tables, precautions and rules regarding the use of chi square have been derived from more than five hundred comparisons between chi square and the exact method. To help in the exact computation of probabilities where that is necessary, four-decimal logarithms of factorials of numbers up to 1000 are given.

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Contents—*Concluded*

Preface

This article was prepared primarily for the guidance of investigators who are grantees of the Medical Division of the National Research Council. Such investigators are commonly concerned with small samples, and, where the data are in the form of measurements, appropriate methods (e.g., Fisher's *t* test) are now commonly applied, at least by those who apply statistical tests at all. On the other hand, where data are classified qualitatively (e.g., as recoveries and deaths, presence or absence of color blindness, rough and smooth colonies of bacteria), many investigators still apply methods, such as chi square and the standard error of the binomial, without appreciating how unreliable these tests are with small samples and skew distributions.

There are available, of course, exact tests, but these are somewhat complicated and laborious, at least to beginners. Indeed, many even find linear interpolation irksome. Some workers therefore recommend the simple tests, to be supplemented by the exact tests where greater precision is needed; but this still leaves the investigator in ignorance of when to apply the exact tests because he does not know how far the simpler crude tests may have led him astray. The better plan, therefore, seems to be: (1) the provision of tables and graphs, based on the more accurate methods; (2) the statement of limits within which the simple tests, especially chi square, can be trusted for problems outside the range of the tables; (3) the presentation, in as simple a form as possible, of methods for the application of exact tests where they are necessary.

As these three objectives became better defined, the present project, which started as an effort to prepare notes on current statistical techniques with medical examples, became in addition an effort to provide suitable tables and graphs, and to experiment with some of the simpler tests in order to define more precisely their reliability and limitations.

In February, 1946, a draft of the notes and specimen tables was issued for criticism by members of the Medical Research Committee and other investigators, and the response was very helpful. Besides affecting details it suggested a change in the plan of presentation. The plan now adopted, and outlined in the Introduction to this article, was devised to meet the wishes of those who, in the words of one investigator, would say: "I have a problem on hand . . . . . . Must I spend a month of free evenings reading a book from end to end several times and mastering all details before deciding how to go about solving the problem? I hope not."

Some workers, of course, object to the kind of plan adopted here, because it appears to put into the hands of an investigator a dangerous weapon without adequate warning, without preliminary explanation of principles, without training in the design of experiments or observations, and without general guidance in the interpretation of the results of statistical tests. On the other hand, many workers have come to appreciate and apply statistical methods by the more direct type of approach. Having obtained statistical help in a

specific problem they have come to enquire about principles and have seen the importance of design, and, having in the first place used tables and graphs that reduce or simplify calculation, they have come to use more elaborate methods in problems that demand them. It is hoped, moreover, that in this article the Introduction, the Comments in the Examples, and the Notes will minimize the dangers of the plan of presentation; although it is doubtful whether any plan could entirely eliminate the beginner's need for personal guidance by someone who has gone farther in statistical methods.

Criticism and suggestions are earnestly requested from those who may examine or use the text, the tables, or the graphs.

Section A—Introduction

This article is so arranged that very little discussion stands between the investigator and the test that he wishes to apply. There is risk in such an arrangement because it suggests that mathematical tests are the most important part of statistical procedure. Of greater importance than the tests are, first, the planning of the experiment or observation so that valid inferences shall be obtainable, and, secondly, the interpretation of the results of the mathematical tests.

Those investigators who are already acquainted with the principles of statistical reasoning, familiar with common terms, and convinced of the importance of random sampling, will presumably use the article chiefly as a means of facilitating their statistical tests. Other investigators will, it is hoped, find in the present section (Section A) a sufficient statement of principles to enable them to start using the tables and tests. The Summary of Examples at the beginning of Section B (p. 12) should help them to find the appropriate table, graph, or test by directing them to a problem like their own.

The Examples sometimes illustrate bad planning, incorrect sampling, unproved assumptions, fallacious deductions, and other errors. These are discussed in the Comments in the Examples, and it is hoped that they will indicate the desirability of a fuller acquaintance with general principles. Section C (Notes) may help investigators to acquire this.

The Examples will, moreover, probably lead the investigator to ask what the tables and formulae are really doing, what is their precision, and what methods are available for greater precision. Such matters also are discussed in Section C.

The Index is intended as a guide to the main topics discussed in all three Sections, and the headings on each right-hand page of the article are arranged to facilitate reference to specific sections, examples, subsections, or notes.

1. Two Types of Data

At the outset the investigator should decide whether his data are, or will be, qualitative statistics (enumeration data) or mensuration data (measurements).

Enumeration Data (Qualitative Statistics)

Enumeration data (qualitative statistics) are records in which individuals, such as persons, animals, blood cells, or bacteria, have been classified according to certain qualities or attributes and the numbers of individuals in the various classes have been recorded. For example, a number of diseased men or animals may be subjected to a certain treatment and then classified under the headings 'death', 'recovery', or 'cure', 'some improvement', 'no improvement'. These may then be compared with another group of individuals, subjected to another treatment and similarly classified. Statistical tests must be applied to show whether we are justified in concluding that one treatment is better than another, and the type of test suitable for this kind of data is different from the type applicable to the other main class of data—mensuration data.

Some laboratory workers, being familiar chiefly with mensuration data, are apt to question the application of precision mathematics to enumeration data unless the material can be sharply classified, e.g., as 'dead' or 'living'. Conceptions of 'mild', 'moderate', and 'severe' tend to differ with different observers and even with the same observer at different times, but this does not mean that judgments based on 'common sense' or 'general experience' can replace statistical tests of such enumeration data.

The statistical tests, far from introducing a spurious precision, commonly show that results, which the untested judgment of the laboratory worker or clinician would consider significant, could easily be accounted for by chance.

The experiments or observations must, of course, be planned to minimize the subjective errors and these then must be investigated, e.g., by the observer testing the constancy of his criteria after a lapse of time; but this must be done also with methods of measurement, which are by no means so objective and unvarying as is often supposed.

Mensuration Data

Mensuration data are records of measurements; for example, the effect of treatment may be measured by change of blood pressure or urinary output. In most cases the actual measurements, their averages, and other derivatives can be used in statistical tests, and the tests differ from those, suitable for enumeration data, in which numbers of individuals and not measurements are used.

This article is concerned with enumeration data, but the principles of statistical reasoning are the same for both types of data, and, as is shown in Examples 37 to 40, measurements can often profitably be treated as qualitative statistics.

2. SOME PRINCIPLES AND DEFINITIONS

Samples and Population

Nearly all forms of medical research involve the use of *samples* (of patients, animals, drops of blood, urines, livers from autopsies, blood pressure readings, and so on), in order to obtain information regarding a *population*, i.e., the material sampled.

Random Samples

Before we can argue from a sample to a population we must be sure that the sample is chosen in the proper way, i.e., in such a way that we can make use of our knowledge of the relations between samples and the populations from which they are drawn. We have a very great deal of such knowledge

when the samples are chosen strictly at random. This does not mean a *haphazard* choice, or the selection of any patients or animals that happen to come along, or a sampling that we believe to be random because we cannot think of any reason why it should not be random.

Random sampling is the kind of sampling that occurs when we deal cards from a thoroughly shuffled pack, or when we mix thoroughly in a box a thousand uniform disks (such as metal-rimmed cardboard labeling tags) and then take out a sample of, say, 20 disks. The factors that determine whether a disk is, or is not, included in a sample are numerous and independent of each other—the physical features of the disk, the various forces and motions imposed on it by gravity, by the other disks, and by the hands that mix the disks. In brief, the occurrence of a disk in a sample, and therefore the composition of each sample, is due to *chance*, which can be defined as *the action of a multiplicity of independent causes.* A *random sample*, therefore, is one whose *composition is determined by chance.* Other definitions are: "a sample chosen in such a way that all individuals in the population have an equal opportunity of being selected", and "a sample chosen according to a rule that is completely independent of the observations to be made on the sample". (For a demonstration of random sampling see Note 1, and for practical techniques see Note 23.)

It is, of course, often valuable, before random sampling, to divide a population into classes. Knowing, for instance, that people (or animals) of different race (or animal stock), sex, and age, often differ in their reactions to disease, to therapeutic treatment, or to experimental treatment, we often divide the original population accordingly. Each of these subclasses thus forms a population to be sampled. In such cases there is at first *purposive sampling* (division into subclasses), but the final sampling must be strictly random if we are to draw valid conclusions.

Statistical tests are methods of drawing such conclusions by showing what allowance must be made for the differences that occur among random samples of the same population, i.e., differences due to chance.

To show how this statement applies to qualitative statistics, it is best to consider first a population whose composition we know, e.g., a thousand persons (500 males and 500 females) and thereby to illustrate the terms 'frequency', 'probability', 'odds', and 'chances'. For concreteness we suppose that each individual is represented by a disk marked 'male' or 'female'.

Frequency

Frequency is the number of individuals of a certain class in a population or in a sample; e.g., 500 males in a population of 1000. In a sample of 10 from that population the males may have a frequency of 4 and the females a frequency of 6. Frequencies are often expressed as percentages, e.g., 50% males; but it will soon become obvious that *statements of percentage frequencies*

in samples, without a statement of actual numbers of individuals, are useless. It is sound advice to *beware of percentages.**

Probability, Chances, and Odds

If there are 500 males and 500 females, when we pick an individual strictly at random our expectation of picking a male is equal to our expectation of picking a female. The *probability* of picking an individual of a certain class by random sampling is defined as the *relative frequency of individuals of that class in the population*, i.e., the frequency divided by the total population. Here the probability of picking a male is 500/1000 = ½ or 0.5. The *chance* of picking a male is one out of two, and the *odds* for male and female are even—one to one.

If, in a population of 1000 persons afflicted by the same disease, 700 recover and 300 die, and we represent this by marking 1000 disks appropriately R or D, the *probability* of picking an R disk is 700/1000 = 0.7, and the probability of D is 0.3. The sum of the probabilities of all classes, here R and D, must be 1. The *odds* in favor of an R disk (against a D disk) are 7 to 3.

A probability indicated by the capital letter, P, is frequently used in tables and tests in order to show whether a result such as we have observed in a certain sampling experiment would be found frequently or rarely by random sampling of a particular population. Definitions of P are given in Section C (e.g., Note 3), but in this Introduction it is probably preferable merely to indicate its general meaning and how it is used in the two main types of problem in qualitative statistics.

Types of Problem

The types of problem in qualitative statistics are mainly: (1) argument from a sample to its population, and (2) comparison of two or more samples in order to decide whether they were probably drawn from the same population or from different populations. Both these types of problem may lead to further questions, e.g.: How large must the sample be before the conclusions desired can be drawn?

The Examples in Section B are classified according to the type of problem; but here we must glance at the general method of reasoning in the two main types.

3. ARGUMENT FROM SAMPLE TO POPULATION

Levels of Significance

Let us suppose that we have 20 patients suffering from a certain disease and treated by a certain method. Nine recover and 11 die. We wish to know whether, by continuing to treat such patients by the same method, our recovery rate might be as high as 70%. We consider our 20 patients as a random sample of the population that we should create by continuing to use the treatment. By methods discussed in Section C, Note 2, we can show that,

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* *A laboratory worker who is used to low percentage errors in techniques of chemical analysis sometimes finds it difficult to realize that a difference of 70 or 80% between samples of enumeration data may have no significance at all.*

if the population recovery rate is 70% recoveries, the probability of 9 or fewer recoveries in a random sample of 20 is specified by $P = 0.017$. Adopting the standards usually applied in such cases we say: "*P is less than 0.025;* therefore the frequency in our sample (9 recoveries in 20, i.e., 45%) is *significantly* different from (lower than) 70%, or the difference between the recovery rate in our sample and 70% is *significant* ".

'Significant' means 'suggestive' or 'indicative' of a real difference. When we give that verdict in the present example we mean that we believe that, if we obtained more and more (or larger and larger) samples of the material represented by the given sample, we should approach a percentage of recoveries that was different from (lower than) 70%. The difference might be either greater or less than the observed difference (70 *minus* 45 = 25%), but we feel justified in believing that *it would not be zero*. Expressed in another way, we believe that chance (random sampling variation) will not account for the difference. In still another form, we say that the true (population) percentage of recoveries is *unlikely* to be as high as 70%.

If our original sample of 20 had contained only 7 recoveries and 13 deaths we should find by the same methods (Section C, Note 2) that, if the population value were 70% recoveries, P for our sample would be 0.001. Again adopting the usual standards we say: "*P is less than 0.005;* therefore the difference is *highly significant* (or *very significant*)". We feel in this case stronger confidence that chance will not account for the difference, and we say that the true percentage is *highly unlikely* (or *very unlikely*) to be as high as 70%.

Finally, *if P is more than 0.025* we say that the difference is *not significant*. This is a verdict of 'not proven'. The evidence is not sufficient to make us believe that a real difference exists. Thus, if our sample of 20 had contained 10 recoveries we should, in a population of 70%, find that P for that sample would be 0.048, which is greater than 0.025. Therefore the difference would not be significant; it might well be due to chance. We could say therefore that the population value was *not unlikely* to be 70% recoveries.

It is shown in Section C, Note 4, that when we say that $P = 0.025$ we mean that only $2\frac{1}{2}\%$ of random samples in a particular population will lie so far (and farther) away from the true (population) value, above or below. Considering also the $2\frac{1}{2}\%$ of samples at the opposite end (below or above) we have a total of 5% of the samples, or 1 in 20; i.e., the odds against finding such samples are 19 to 1. For $P = 0.005$ the corresponding figures are $\frac{1}{2}\%$ of the samples below the true value and $\frac{1}{2}\%$ above—a total of 1% or odds of 99 to 1.

To contrast the two levels of significance we speak of the one with $P = 0.025$ as the $2\frac{1}{2}\%$ level of significance, and of the one with $P = 0.005$ as the $\frac{1}{2}\%$ level of significance.

For discussion of the reasonableness of these significance conventions see Section C, Note 5.

Confidence Limits

A given sample may belong either to (1) a population with a higher percentage than the percentage shown by the sample, or to (2) a population with a lower percentage than the sample percentage; but, using the probability criteria ($P = 0.025$ and $P = 0.005$), we can state, with confidence corresponding to those probabilities, the limits beyond which we do not believe that the true (population) percentage lies. Tables I (A and B) and II enable us to find such limits. For instance, if we have a sample of 20 patients with 9 recoveries and 11 deaths, we turn up Table IB under "Number of A's in sample = 9", and look along the line $N = 20$. Under "Upper limits, $P = .025$" we find 68.5%. This means that the upper confidence limit at the $2\frac{1}{2}$% level ($P = 0.025$) is 68.5%. On the same line we see that the upper limit at the $\frac{1}{2}$% level ($P = 0.005$) is 74.3%. We can express these findings in words by saying that the proportion of recoveries, in the population of which our sample is a random sample, is *unlikely* to be more than 68.5% and *very unlikely* (or *highly unlikely*) to be more than 74.3%.

The lower limits are likewise shown, and we can say that the population percentage is unlikely to be outside the range or *confidence belt*, 23.0 to 68.5%, but, if we adopt the usual rule, we are not prepared to estimate it any closer than that. It may lie anywhere within that belt. However, in some cases it may be sufficient or desirable to accept a narrower belt, 29.3 to 61.5% at the 10% level ($P = 0.1$), but our confidence here is low. (For further discussion see Section C, Note 6.)

4. COMPARISON OF TWO OR MORE SAMPLES

Arrangement of Data

Let us suppose that a random sample of nine individuals from a certain population, e.g., a population of young adult male human beings with a certain disease, receives Treatment V, and another random sample of six from the same population receives Treatment W. The observations are recorded in a fourfold table in which actual numbers, *not percentages*, are given:

Treatment	Recoveries (R)	Deaths (D)	Total
V	2	7	9
W	5	1	6
Total	7	8	15

Many different kinds of data can be presented by such a table. For example, among nine women, attacks of a certain disease might be severe in seven, mild in two, whereas among six men they might be severe in one and mild in five; or a certain muscle might be present in only two of nine male white cadavera, and in five of six male Negro cadavera. A table that shows the *joint occurrence* of two sets of attributes or qualities (e.g., type of treatment

and outcome of disease, sex, and severity of attack) is a *contingency* table (Latin, *con*, together; *tangere*, to touch). Each of the two sets of attributes may have two or more components, e.g., 'mild', 'moderate', 'severe'. The simplest form, as in the present example, is a fourfold contingency table. For analysis, its contents can be looked on either as two 'treatment' samples divided according to outcome (recovery or death) or as two 'outcome' samples divided according to treatment. The results of the analysis will be the same.

Methods of Analysis

Methods of analysis of such data are shown by numerous examples in Section B. For very small samples (up to 20 individuals) the results can be obtained directly from Tables IV or V, and for larger, but equal, samples Table VI provides some information. In other cases simple mathematical formulae are used. Whatever particular technique is appropriate, the underlying reasoning can be illustrated by reference to our samples (Treatments V and W) as follows.

Let us suppose that there is no real difference between the effects of Treatments V and W. If, then, we were to take more and more samples we should find that some showed V apparently better than W, while others showed W apparently better than V, but when we combined the results we should find that the recovery rates for the two treatments became more and more alike, and we should become more and more convinced that, in respect of recovery rate, the samples were all from one population. Let us therefore apply this supposition to our actual samples of nine and six patients, and find out how frequent or how rare would be the occurrence of such differences as we have observed.

As before, we use the probability P as a measure of this and we adopt the same standards of significance. Thus, it will be shown in Example 15 that, if our samples came from the same population, P would be greater than 0.025, i.e., we should expect, as a result of chance alone, to find more than $2\frac{1}{2}\%$ of samples showing as much, or more, evidence in favor of Treatment W.

We therefore say that there is *no significant difference* between the two samples. We have not sufficient evidence to say that the Treatment W is better than Treatment V, and, for all we know, Treatment V may be better than Treatment W.

If P had been between 0.025 and 0.005 we should have said that there was a *significant difference*, and we could express this result in other ways also:

(1). We do not believe that chance (random sampling variation) is responsible for the difference.

(2). There is a significant *association* between difference in treatment and difference in recovery rate (proportion of recoveries).

(3). There is a significant *heterogeneity* or a significant *lack of homogeneity* between the samples, i.e., we believe that they are not from the same 'material' or population.

Levels of Significance

If our samples had shown a difference in favor of Treatment V we should have applied the same standard, $P = 0.025$, referring to the $2\frac{1}{2}\%$ of samples in which differences of that type occurred. As in the argument from sample to population (p. 8) we are therefore excluding a total of 5% of samples, with odds of 19 to 1 against finding such samples (V better than W, or W better than V) if there is no real difference between the effects of the two treatments; and similarly, with $P = 0.005$ we are excluding a total of 1% of samples (odds of 99 to 1).

One-sided Comparisons

Sometimes, of course, we are concerned with differences in one direction only. For example, we may try the effects of a treatment by comparison with a sample of control animals. We may know that the treatment cannot do any harm, and we ask: Is treatment *significantly better* than no treatment? This is a 'one-sided' comparison, in contrast to the 'two-sided' comparison of Treatments V and W. As is shown in Section C, Note 5, P values of 0.05 and 0.01, instead of 0.025 and 0.005, would be the logical criteria in one-sided comparisons, but this complication is often unnecessary, and we shall in most instances treat one-sided comparisons as if they were two-sided. We are thereby merely raising our standards of significance somewhat for the one-sided comparisons.

(For further discussion of contingency tests see Section C, Note 12.)

5. Random Sampling

Importance of Random Sampling

The object of the experiment discussed in Subsection 4 was to see whether differences in treatment would or would not create a significant difference between the samples—a difference greater than could be reasonably accounted for by random sampling. Therefore obviously it is no use analyzing the data, or conducting the experiment at all, unless we have allocated the treatments to the two samples of patients by *strictly random methods*. When we are not experimenting but observing, e.g., the sex incidence of severity of disease, or the racial frequency of a muscle, it is often difficult to generalize because we often cannot be confident that a hospital or anatomy laboratory has provided us with random samples.

Simplicity of Techniques

Although proper techniques are now commonly used in many types of investigation that are very like those of clinical and laboratory medicine, some medical investigators still feel that the techniques are needlessly complex and artificial. A little experience will show how simple and practical they are—see Section C, Note 23, for examples.

Equality of Numbers

Random sampling is sometimes easier if equal numbers of individuals are chosen for each sample, but the most important reason for choosing equal

samples is that, for a given total number of individuals, equal samples provide
the most sensitive tests, i.e., they give the greatest amount of information.
However, unequal samples are sometimes desirable. For example, a certain
form of treatment may be expensive, time-consuming, difficult, disagreeable,
or even dangerous to the subjects. It may then be desirable to treat fewer
individuals by this method and use more in the other group.

Bias and Sample Size

Many workers believe that small samples are specially apt to be biased·
For one who holds this belief, the best way, probably the only way, to
appreciate its fallacy is to become acquainted with statistical reasoning and
methods. He will then see that the only difference between small random
samples and large random samples is that the small samples do not give as
precise information regarding the sampled population, and that, however
small the sample may be, the degree of this precision can be estimated. He
will further realize that unequal samples, when properly treated, do not
introduce bias.

Section B—Examples

SUMMARY OF EXAMPLES

1. Argument from a sample to a population.
    (1) N = total number of individuals in sample. Two classes—A,
        not-A. (Classify as 'A' the individuals that form not more than
        half the sample.) Example 1 for general methods, interpretation,
        and comments.
        (a) Number of A's in sample: 0 to 20, whatever the value of N.
            Examples 1 and 2. Table IA and IB. In addition:
            (i) N in sample between two N values in the tables.
                Examples 3 and 4.
            (ii) To answer the question: Does the sample indicate
                that A's form less than half the population, i.e., that
                not-A's are in the majority? Examples 5, 6, and 24.
            (iii) Number of A's in sample = 0. Example 7.
        (b) Number of A's in sample more than 20. Example 8. Table
            II; Graphs 1 to 3 (= Figs. 6 to 8). In addition:
            (i) N or percentage of A's in sample between values in
                Table II. Examples 9 and 10.
            (ii) To answer the question of majority and minority as
                in (a) (ii), above. Example 11.
            (iii) Percentage of A's in sample less than 5. Example
                12. Tables II and III; Graphs 4 to 6 (= Figs. 9
                to 11).
    (2) More than two classes in sample—A, B, C, etc. Example 13.
        Chi square.

2. Comparison of samples.

    (1) Comparison of two samples, each divided into two classes (a four-fold contingency table).

        (*a*) Two equal samples, from 1 to 20 individuals in each. Example 14. Table IV.

        (*b*) Two unequal samples containing up to 20 individuals in one sample, up to 19 in the other. Examples 15 to 18. Table V.

        (*c*) Two equal samples containing 20 to 1000 individuals in each. Example 19. Table VI.

        (*d*) Any pairs of samples, equal or unequal, containing more than 20 individuals in either. Examples 20 to 23, 25, 26. Chi square.

    (2) Comparison of more than two samples and of samples with more than two classes (contingency tables larger than fourfold). Examples 27, 28, and 29. Chi square.

3. Combination of information from two or more samples. Examples 30 and 31.

4. Confidence limits for differences between samples. Example 32.

5. Sizes of samples required.

    (1) In estimation of confidence limits. Examples 33 and 34.

    (2) In comparison of samples. Examples 35 and 36.

6. Measurements treated as qualitative statistics. Examples 37 to 40.

1. Argument from Sample to Population

(1) two-class samples

Example 1

The same numerical problem can take many forms. Five variants are given.

(1). A physician treats 25 patients suffering from a certain disease and 15 die (60%). In this disease the mortality, based on reports of thousands of cases, is 40%. What right has the physician to think that his sample is exceptional?

(2). Twenty-five sailors, tested by motion in a rocking machine, were pronounced susceptible to motion sickness, i.e., they always developed symptoms when so tested. A certain drug was administered, the test was repeated, and 15 (60%) were found to be immune to the motion. What confidence can we have that the true percentage, approached by performing the same experiment on thousands of similarly susceptible men, would be more than 40%? In more general terms, between what limits should we expect the true percentage to lie?

(3). A certain inoculation invariably produces disease in unvaccinated rats. In 25 rats, previously vaccinated, 10 (40%) develop the disease and 15 (60%)

do not. The experimenter says: "If this vaccine is unlikely to protect more than 75% of the rats, I do not wish to try it on hundreds of animals. I prefer to set about developing another vaccine."

(4). A pharmacologist injects a substance in 25 dogs and produces an effect, such as vasoconstriction or death, in 15 of them. He asks: "What is the error in this estimate (60%), i.e., what might be the percentage if I performed the same experiment on more and more dogs of the same kind?" This variant indicates how the methods to be discussed here are applicable in biological assay, including toxicity tests.

(5). The combined pedigrees of several families show the occurrence of a certain disease or defect in 15 of 25 children. If a certain genetic mechanism is at work the ratio to be expected is 3 : 1. Does the sample of 25 agree with this hypothesis?

Method

In each variant there is a sample of 25 with 15 in one class and 10 in the other. Call the class that contains less than half the total '*A*' because, to save duplication, Tables IA, IB, and II are arranged for numbers up to half the total. The number of *A*'s is 10; therefore use Table IB—number of *A*'s: 1 to 20. (For procedure where the number of *A*'s is over 20 see Example 8.) Under heading "No. of *A*'s in sample = 10", find the confidence limits along the line $N = 25$. (For procedure where N lies between two values in the table see Examples 3 and 4; for N greater than 1000 see Example 4.)

The wide limits ($P = 0.005$) are 16.8 and 67.0%, the medium limits ($P = 0.025$) 21.1 and 61.3%, and the narrow limits ($P = 0.1$), which may be needed for some purposes, are 26.5 and 54.8%.

Since the problem is stated in terms of the other class (not-*A*) subtract each of these from 100%, to give:

Wide limits ($P = 0.005$)—83.2 and 33.0%,

Medium limits ($P = 0.025$)—78.9 and 38.7%,

Narrow limits ($P = 0.1$)—73.5 and 45.2%.

Interpretation

Variant (1).—The true mortality may be between 38.7 and 78.9%, without the sample being exceptional. If the physician thinks that his sample (60% mortality) indicates a mortality higher than 38.7% he is adopting standards that will lead him astray on more than 5% of the occasions on which he estimates upper and lower limits, and if he thinks that his sample indicates a mortality higher than 45.2% he is adopting standards that will vitiate more than 20% of such estimates (see Section A 3).

Variant (2).—Accepting the usual standards of judgment, we are not justified in assuming that, if thousands of similar men were examined, more than 38.7% of them would show immunity after the drug; and we are even less justified in assuming an immunity of more than 40%.

Variant (3).—There is no significant difference between the sample frequency (15 in 25) and 75%, for there is no adequate reason to suppose that the vaccine will protect fewer than 78.9% of such rats (or more than 38.7%).

Variant (4).—The pharmacologist can state, with confidence represented by odds of 19 to 1, that the true percentage is unlikely to be outside the range of 38.7 to 78.9, and he can state, with confidence represented by odds of 99 to 1, that it is unlikely to be outside the range 33.0 to 83.2.

Variant (5).—The ratio 3 : 1 means 3 out of 4, i.e., 75%. There is no significant difference between the sample frequency (15 in 25) and 75%. The observation does not disagree with the hypothesis.

Comments

It is perhaps desirable to point out that two types of problem have been illustrated here:

(*a*). A test of the significance of the difference between a sample and a (real or hypothetical) population value—Variants (1) and (5).

(*b*). An estimation of confidence limits—Variant (4). The other variants have elements of both types.

To the investigator, however, the distinction may be unimportant, because, as has been shown, the desired information has been obtained by the same technique for each variant.

Comment on Variant (1).—Mortality in 25 patients. A percentage or ratio *derived from a sample*, even if the sample contains thousands of individuals, is not the same as a *postulated* percentage or ratio, as in (2) or (5). The postulate may be purely hypothetical or may be derived from observed phenomena (e.g., dice-throwing or genetic mechanisms), but it is always exact. It can be looked on as a 'true' value, derived from a population that is infinite, i.e., as large as we care to make it; whereas an actual (finite) sample, however large, can only give an estimate. The distinction is often unimportant if the sample contains several thousands, but percentages are frequently given in textbooks and other secondary sources without sample size, although the latter, on further search, turns out to be quite small.

The proper method of treating the physician's sample of 25 would therefore be *comparison with the sample* that gave the reported 40% mortality. However, as a preliminary step we can treat the 40% as a postulated 'true' value, as was done above, and then:

(*a*). If the sample shows no significant difference from this postulated value we can be assured that comparison of samples would show still less evidence of a difference, because the postulated 'true' value is equivalent to the value for an 'infinitely large' sample.

(*b*). If the observed sample shows a significant difference from the postulated percentage, we must find the size of the reported sample. If it is 1000 or more it will often be safe enough to take its frequency as a population frequency; but it is safer, and not difficult, to compare the observed sample with the reported sample. (See also Examples 3 and 26.)

(c). If there is a significant difference between the samples *we must be extremely cautious in drawing conclusions regarding the causal factors.* For example, hospitals tend to receive more severe cases than those treated in private practice, and their data vary with changes in the customs of people regarding hospitalization for a certain disease and with changes in availability of hospital space. Statistics from Departments of Health suffer from many weaknesses, e.g., differences in diagnoses between different individual physicians, between the physicians of different regions, and at different periods.

Comment on Variant (2).—The design of the experiment on motion sickness is unsatisfactory and thereby illustrates an important feature common to a wide variety of experiments. To be very confident that a man who had shown susceptibility, even in all previous tests, would show it if he did not receive the drug, would require many men and perhaps many tests on each. The results of these experiments would have to be expressed with an estimate of error (as a confidence limit or in some other form), and this estimate should be allowed for in the test of the drug. *A well-designed experiment contains in itself its estimate of error*, and a procedure such as the following could be suggested:

(a). If it is desired to select the apparently more susceptible men, do so by a preliminary test.

(b). Allocate in advance to the selected group, *strictly at random*, treatment and nontreatment. This takes care of all such factors as variable degrees of susceptibility, activities before the test and time of day when the test is made. Even loss of men owing to unforeseen circumstances (unless associated with their susceptibility to motion sickness) does not introduce a bias, although it removes the advantage of equal numbers.

(c). Compare the two samples as in the Examples indicated under that heading in the Summary of Examples (Subsection 2).

(d). If there is a significantly greater immunity in the drug-treated men, estimate for them the confidence limits as shown in the present example. The answers will show estimates of immunity *after* the drug, not necessarily wholly *due to* the drug, even if the nontreated men, in the particular experiment, all developed motion sickness.

Comment on Variant (3).—Vaccination of rats. See Comment on Variant (2). The same experimental design, with strictly random sampling, should be used, to randomize the variations due to such factors as constitutional differences between rats of different litters, differences in response to inoculation, and numerous environmental differences, e.g., in cage positions. The investigator may say that such factors can have only a slight effect, if any. He nevertheless recognizes that rats differ in their response to inoculation and vaccination, but is ignorant of many of the factors responsible. Randomization automatically distributes these factors, so that the differences between the vaccinated animals and controls are, except for the vaccination, due to chance.

Comment on Variant (4).—Vasoconstriction in dogs. The data are enumeration data (qualitative statistics—vasoconstriction present or absent), which may be sufficient for the investigator's needs, perhaps as a preliminary survey; but where measurement is possible (e.g., amount of injected substance, degree of vasoconstriction) mensuration data are obtained and, in general, they give more information than do enumeration data.

Comment on Variant (5).—The sample does not disprove the hypothesis— the genetic mechanism that is responsible for a 3 : 1 ratio. But this does not prove that the hypothesis is correct. Reference to the confidence limits already obtained will show that:

(a). The hypothesis of a 2 : 1 ratio (66.7%) is not disproved by the sample, because, even for $P = 0.1$, the true value may be 73.5%.

(b). A 13 : 3 ratio (81.25%) is unlikely (P less than 0.025), but not very unlikely (P less than 0.005).

(c). A 1 : 2 ratio (33.3%) is unlikely; and is almost as low as the lower limit, 33.0% for $P = 0.005$.

Where there is a limited number of hypotheses as here, we can, by increasing the sample size, disprove one after another with the degree of confidence that we desire, until only one remains, which therefore is the most likely to be correct.

Example 2

An anatomy textbook states that the posterior ethmoidal nerve is found in only 30% of orbits. The sample size is not given, as it ought to be if the percentage is to have any value, but the original report revealed that it was 10. How far is the generalization (30%) justified?

Method

In Table IB, under "Number of A's in sample = 3", for $N = 10$ find the medium confidence limits ($P = 0.025$)—6.7% and 65.2%.

Interpretation

For the various populations, of which this could be a random sample, the percentage might lie anywhere between the indicated limits.

Comment

Although these particular data on nerve frequency are intrinsically of little importance, they illustrate a weakness that should be guarded against in more important problems. In bilateral organs or parts (kidneys, orbits, limbs, etc.), because of the varying tendency toward similarity of the two sides, reports, and calculations based on them, should make the distinction clear. Thus, if there were here five cadavera (10 orbits) with two nerves in one and one nerve in a third, this would not afford an estimate for a random sample of orbits unless there were no tendency to bilateral symmetry of nerves.

Example 3

In a class of 98 male medical students eight are color blind. Accepting a textbook statement that 4% is the common incidence of color blindness in males, are we justified in expressing surprise at this '100%' excess?

Method

In Table IB, under "Number of A's in sample = 8", find the lower confidence limit ($P = 0.025$) for $N = 90$ (3.9%) and $N = 100$ (3.5%). For $N = 98$ the limit must be about 3.6%. Our sample shows no significant difference from a 4% population value.

Comment

For the danger of using reported percentages as if they were true (population) percentages, see Example 1—Comment on Variant (1), and Example 26. If the test had shown a significant difference and we wished to pursue the problem, we should find the records of the samples underlying the textbook statement, and compare those samples with our own. If this showed a significant difference we should be justified in seeking causes, but we should bear in mind three things:

(1). We may not have been counting the same thing as the other observers, i.e., their definitions of, or tests for, color blindness may have been different from ours.

(2). Let us suppose that a physiologist found in his class of 98 students a frequency of color blindness that would occur by chance once in, say, 30 such samples from a population with 4% frequency. With a new class of the same size every year he should expect to meet such a chance occurrence once in a teaching career of 30 years.

(3). "The 'one chance in a million' will undoubtedly occur, with no less and no more than its appropriate frequency, however surprised we may be that it should occur to *us*"—Fisher (10).

Example 4

The same problems as in Example 1, but with $N = 102$, number of A's in sample = 10. In that section of Table IB, the required confidence limits must lie between those for $N = 100$ and those for $N = 110$. In many cases this will be sufficient information.

If a somewhat more precise estimate is desired, note that 102 is one-fifth of the way from 100 to 110, and interpolate mentally, giving, for example, about 3.7% for the extreme lower limit and a little under 20% for the extreme upper limit.

If a still more accurate estimate is needed, divide 102 (the observed N) by 100, to obtain 1.02, and divide this into the values for 100, to obtain 3.7% for the extreme lower limit and 19.8% for the extreme upper limit.

If N in the sample is, say, 108, i.e., nearer to 110 than to 100, divide 108 into 110, to get 1.02, and multiply 1.02 by the tabulated values for $N = 110$.

If N is halfway between two tabulated values of N, either of the above methods of interpolation can be used.

Note how these rules apply at the foot of the table. One can find the limits for $N = 1000$ by dividing 500 into 1000, to give 2, and then dividing the values for 500 by 2.

If N in the sample is greater than 1000, divide the values for 1000 by N/1000.

Example 5

Among eight bubonic plague patients who had meningitis six were male and two were female (18). Is it justifiable to say that males seem to be more susceptible?

This is an example of a common form of misleading data (see Comments), but we can use the figures to show how to answer the question: Is there any evidence of a *significant majority* of males?

Method

Let A be the number of females. In Table IB, under "Number of A's in sample $= 2$", for $N = 8$ the upper confidence limits are 53.9% ($P = 0.1$) and 65.1% ($P = 0.025$). There is therefore no reason to believe that further investigation would not show that the females were in the majority.

Comments

Whether the difference was significant or not we could tell nothing about the relative sex incidence, because we do not know the proportions of the sexes in the population of plague patients from which the sample was drawn. The proper method of analyzing would be to divide the plague patients into two samples—(1) males: number with meningitis, number without meningitis; (2) females: number with, number without. Comparison of the two samples would show whether there was a significant association with sex.

Data presented as in this example can be misleading. For instance, in 312 patients with hookworm disease 55.8% were men and 44.2% were women (12). If one takes these data as suggesting that, in general, the male incidence is higher than the female, one is making the unwarranted assumption of male–female equality in the general population and in the part of it available for this survey.

Another example of the same kind of presentation occurs in a report on the incidence of tuberculosis in the naval service (17). Of the total number of cases, 5.1% were females, 8.6% were officers, 52.5% were seamen, etc., 12.9% were engine room workers, and the remainder were in other groups. Without a statement of the total numbers employed in each group no conclusion can be drawn regarding the relative incidence in the different groups; and yet a reader, meeting such data, is very apt to draw some such conclusion.

Example 6

Two treatments, V and W, have been applied to each of 10 animals, e.g., two methods of treating experimental wounds or burns.　The results are:

　　　Animal No.
　　　　　　1 — No appreciable difference
　　　　　　2 — W better than V
　　　　　　3 — W better than V
　　　　　　4 — No appreciable difference
　　　　　　5 — W better than V
　　　　　　6 — V better than W
　　　　　　7 — W better than V
　　　　　　8 — W better than V
　　　　　　9 — No appreciable difference
　　　　　10 — W better than V

Is there evidence that W is really better than V, i.e., is there a significant majority in which W is better?

Similar data might represent 10 specimens of a certain microorganism, each divided for growth on two plates of culture medium, a growth-inhibitor having been added to one plate of each pair (or one growth inhibitor to one plate and another to its fellow).

Method

Nos. 1, 4, and 9 tell us nothing about the possible differences.　In the remaining seven, six show W better than V, and one shows V better than W. In Table IB, under "Number of A's in sample = 1", for $N = 7$ find the upper limit ($P = 0.025$)—57.9%.

Interpretation

For all that the sample proves, if we increased the number of such observations we might find that, among those that showed an appreciable difference, V was better than W in more than 57%—the opposite of what the sample suggests.　Therefore there is no adequate proof that W is really the better treatment.

Comments

(1). It is desirable where possible in such cases to apply methods of measurement, e.g., number of days to wound healing, size or number of bacterial colonies, or even a method of grading the effects, e.g., by 1, 2, 3, 4, etc., provided that the system of grading is uniform.　Methods of testing mensuration data can then be applied and may display significant differences where enumeration methods do not.　For example, in the animal where V was better than W the difference might only be slight, whereas W might be much better than V in several animals.

The test applied above would, however, be appropriate where there is no satisfactory system of measurement, or to compare gross or qualitative effects, e.g., colonies and no colonies on culture media.

(2). It has already been shown that there is no significant difference between our sample of seven and a true (population) value of 57%—a majority of 7% in favor of V. Therefore, *a fortiori*, there is no significant difference from a population percentage of 50—equal effects of V and W. It might be asked: "If there were no real difference between V and W, how often would samples showing as great an apparent difference in favor of W be found by chance?" By the method of Section C, Notes 2 and 3, it can be proved that P for our sample $= 0.0625$, i.e., far greater than 0.025. We should expect 6.25% of samples of seven to show as great a difference as observed, or greater.

(3). The data might represent 10 pairs of animals, Treatments V and W having been allotted at random within each pair; but one must *beware of artificial pairing*, because much information can be thereby lost or obscured. Pairs of animals from each of 10 litters could be used, provided that there was good reason to believe that their reactions to the particular disease or experimentally induced condition, for which Treatments V and W were to be tested, would be more alike than the reactions of animals selected purely at random. Again, in testing a vaccine as a possible preventive of infection in human beings, it may be desirable to make use of the fact that within a family there is similarity in risk of exposure to the disease and in other environmental features. Within each family, of course, the treated and control members must be chosen strictly by a random sampling method.

(4). The hypothesis tested here was that, among animals showing an appreciable difference under the two treatments, the true (population) ratio, (W better than V) : (V better than W), is 1 : 1. Animals 1, 4, and 9 were omitted because the hypothesis does not tell us how many animals to expect in which no appreciable difference could be detected. The whole sample of 10 would, however, be taken into consideration if other questions or hypotheses were to be considered; e.g., if we asked: "What are the confidence limits for the percentage showing an appreciably better result with W?" In that case the other class would contain (1) those that showed a better result with V, and (2) those that showed no appreciable difference.

(5). The same figures as in this example might have been obtained by comparing animals, subjected to a certain treatment, with their untreated litter-mates. If we knew that the treatment could not be worse than no treatment, we should be concerned only with samples in which treatment was apparently better than no treatment—a one-sided comparison, mentioned in Section A4, and discussed in Section C, Note 5. The test of significance, applied to the two-sided comparison in the present example, need not be altered, and if the observer wished to use $P = 0.05$ as a criterion there would still be no significant evidence that treatment was better than no treatment, for $P = 0.0625$.

Example 7

A certain blood substitute, transfused into 80 patients, produced no unfavorable reaction. It is possible that, transfused into all the present and future inhabitants of the world, it would produce no reaction, but we wish to know

how much confidence we may derive from the sample of 80, i.e., what proportion of individuals might react if we transfused this substance into more and more of the people represented by our 80 as a random sample.

Method

. The number of A's in the sample is zero. Therefore use Table IA. For $N = 80$, the upper limits are 4.5% ($P = 0.025$) and 6.4% ($P = 0.005$).

Interpretation

Extensive further investigation might reveal that the percentage of persons presenting unfavorable reactions was as high as 4.5%, although we can feel reasonably confident that it is unlikely to be more. In a problem of this kind, however, we are more inclined to demand the greater assurance offered by the $\frac{1}{2}$% limit ($P = 0.005$) and, if so, we must say that we do not trust the percentage of unfavorably reacting individuals to be less than 6.4.

Comments

(1). These statements apply to a population of which the 80 patients would be a random sample. By increasing the size of the sample from the same type of patient (the same population), if we still found no reactors, we could reduce the upper confidence limit as far as we wished; but it would obviously be more useful to increase the variety of patients at the same time as we increased our sample size.

(2). Table I shows that, even if we have no A's in a sample, we require a sample of six before we can be reasonably confident ($P = 0.025$) that the population contains fewer than 50% A's, and we require a sample of eight before we can be very confident of this ($P = 0.005$).

Example 8

In the problems of Example 1, let us suppose that the total number in the sample is 80, with 48 (60%) in one class and 32 (40%) in the other. Calling the less numerous class "A", we find 32 A's, and as this is more than 20 we use Table II, or Graphs 1, 2, and 3 (= Figs. 6, 7, and 8).

Under "Percentage of A's in sample = 40" find $N = 80$ and read off the limits. Alternatively, in Graphs 1, 2, or 3, find $N = 80$ at the foot, run up to the lines for 40% and find the confidence limits by reference to the left-hand scale.

When the values in the observed sample are not directly given in Table II, interpolation in the graphs will often be sufficient—see Examples 9 and 10.

Example 9

When confidence limits are required from a sample containing more than 20 A's (A being the less numerous class, as usual), Table II is to be employed as in Example 8, but in some cases interpolation is necessary. If N is intermediate between two values in Table II, but the percentage of A's is directly given by the table, proceed as in the following example.

$N = 95$; number of A's $= 38$, i.e., 40%. In Table II find "Percentage of A's in sample = 40". The required limits must be between those for $N = 90$

and those for $N = 100$; and such an approximation is often sufficient. For a more precise estimate, use Graphs 1, 2, or 3 (= Figs. 6, 7, or 8). Thus, for the extreme lower limit ($P = 0.005$) in Graph 1 run up the vertical line for $N = 95$ until it strikes the line of lower limits (continuous line) for 40%, and read off the required value, i.e., 27.5%.

For most purposes interpolation in the table will probably be unnecessary, but, if it is desired, simple (linear) interpolation is sufficiently accurate. Thus, we take the limits for $N = 95$ about halfway between the tabulated values for $N = 90$ and $N = 100$. For the extreme lower limit the difference between the tabulated values is $27.8 - 27.2 = 0.6$. Half of $0.6 = 0.3$. Therefore for $N = 95$ the limit is approximately $27.8 - 0.3 = 27.5\%$.

If N were 92, the required limit would be taken as one-fifth of the distance from 27.2 to 27.8, and similarly for other intermediate values of N. (If the percentage in the sample is intermediate between two tabulated values, see Example 10.)

Example 10

N, i.e., total number in sample $= 52$; number of A's in sample $= 22$, i.e., percentage of A's in sample $= 42.3$. The confidence limits cannot be read directly from Table II, but for many purposes the graphs will be sufficient. Thus, to find the lower limit at the $\frac{1}{2}\%$ level ($P = 0.005$) in Graph 1 (= Fig. 6), use the continuous (lower limit) lines for 40% and 45%. At the foot of the graph locate the position of $N = 52$ and run up to a point nearly halfway from the 40% line to the 45% line. Reading the level of this point on the left-hand side of the graph it appears to be above 25% but not quite 25.5%. Further, by noting that the sample percentage is situated at $2.3/5$, i.e., 0.46, of the distance from 40 to 45, it was found possible, with the aid of a millimeter scale, to estimate the required limit as 25.3%.

Alternatively, using Table II we note the following values for lower limits:

N	Percentage of A's in sample	
	45%	40%
50	27.3	23.0
55	28.1	23.7

This is sufficient to show that the required value is between 23 and 28%, or approximately 25%. Such information may be enough in many problems; but for a more precise result we can proceed as follows by simple interpolation:

The N in our sample, 52, lies at $2/5$ of the distance from 50 to 55. Therefore, under 45% the required value should be at about $2/5$ (or $4/10$) of the distance from 27.3 to 28.1, i.e., 0.4 of $(28.1 - 27.3) = 0.4$ of $0.80 = 0.32$, i.e., with only one decimal figure, 0.3. This, added to 27.3, gives 27.6 for the 45% column.

Similarly, for the 40% column the value is $23.0 + 0.4$ of $(23.7 - 23.0) = 23.0 + 0.28 = 23.3$ to one decimal place.

Our sample percentage, 42.3, lies at 2.3/5 (i.e., 0.46) of the distance between the two values found above,—0.46 of the distance from 23.3 to 27.6. It is therefore $23.3 + 0.46$ of $(27.6 - 23.3) = 23.3 + 2.00 = 25.3\%$, as was found by interpolation in the graph.

Example 11

Intranasal inoculation of 57 monkeys with poliomyelitis virus (8) gave the following results:—

> Arms first paralyzed: 25 animals,
> Legs first paralyzed: 27 animals,
> Arms and legs paralyzed at about the same time: 5 animals.

Arm paralysis indicates involvement of cervical segments of the spinal cord; leg paralysis indicates involvement of lumbar segments. Is it justifiable to state that "the virus can, and does in more than half the cases, produce its first manifestations in the lumbar segments?"

Method

The third class (five animals) tells us nothing about unequal frequencies of involvement. Of the remaining 52 animals, 25 showed arm paralysis first, 27 showed leg paralysis first. The number of A's (25) is more than 20; therefore use Table II and Graphs 1 to 3 (= Figs. 6 to 8) first expressing 25/52 as a percentage—48.08%; $N = 52$.

If the true value of A (arm paralysis first) could be *more* than 50% without transgressing the limits that we usually allow for the effects of chance, the true value for not-A's (leg paralysis) could likewise be *less* than 50%.

Graph 2 (= Fig. 7) shows that the upper limit $(P = 0.025)$ lies between 62 and 63%. Graph 3 (= Fig. 8) shows that even for $P = 0.1$ the upper limit is about 58%.

In Table II the required upper confidence limit for A's would be found between the values for sample percentages 50 and 45, with N between 50 and 55. In this instance, however, the verdict is obvious because, even if the percentage of A's in the sample were 45 instead of 48.08, and even if we had information from 55 animals instead of only 52, the population percentage could be as high as 58.9, i.e., the percentage of not-A's (legs paralyzed first) could be as low as $100 - 58.9 = 41.1$.

Interpretation

The sample does not show a significant majority of animals with leg paralysis earlier than arm paralysis; i.e., there is no adequate evidence of a tendency for legs to be paralyzed first.

Even if we were content with a much lower degree of confidence $(P = 0.1)$ we could not say that we believed that the true percentage of not-A's (leg paralysis first) would be more than about $(100 - 58) = 42\%$.

Comment

An opposing hypothesis regarding the mode of spread or action of the virus might entail the earlier paralysis cf the arms in a majority of animals, e.g., that the proportion of A's should be 60%. The sample does not disprove that hypothesis because the upper limit ($P = 0.025$) is over 60%.

Example 12

Table II carries the percentage of A's in the sample down to 0.1, i.e., 1 per 1000, and Graphs 4, 5, and 6 (= Figs. 9, 10, and 11) cover the regions from 5% to 0.1%.

In these regions of the graphs, N becomes larger than is common in small-scale medical research, and less detail is given than in Graphs 1 to 3 (= Figs. 6 to 8). Therefore, although the methods of Examples 8 to 11 can be used, greater precision will sometimes be necessary, and sometimes very large samples (greater than 20,000) with percentages of less than 0.1 will be met. Table III, derived by interpolation in Table VIII 1 of Fisher and Yates (11), will help in such cases, and can be used for even higher percentages (up to 10%, with N greater than 200). For example: $N = 460$; number of A's = 22. Therefore percentage of A's = 4.783. Required: the upper confidence limit ($P = 0.005$).

Method

The procedure can be divided into four stages.

Stage (1).—Express the percentage as a decimal fraction, i.e., the sample probability, $p = 0.04783$. Then $q = 1 - p = 0.95217$.

Stage (2).—Estimate the standard deviation $\sqrt{Npq}$, which is easier to estimate when expressed as $\sqrt{Aq}$, where A is the number of A's in the sample, here 22. Four-figure logarithms are adequate.

Log A = log 22 = 1.3424. Log q = log 0.95217 = $\bar{1}.9787$.

Log Aq = $1.3424 + \bar{1}.9787 = 1.3211$. Log $\sqrt{Aq}$ = $\frac{1}{2}$ log Aq = 0.6606.

Do not convert from logarithms at this stage.

Stage (3).—Use the general formula for any confidence limit:

$A \pm F\sqrt{Aq}$ + appropriate correction term from Table III.

F is a factor depending on the level of the limit required:

For $P = 0.005$, $F = 2.5758$; log $F = 0.4109$.

For $P = 0.025$, $F = 1.9600$; log $F = 0.2922$.

For $P = 0.1$, $F = 1.2816$; log $F = 0.1077$.

The factors are obtained from the normal curve (Section C, Note 7). The values given here were derived from Table I of Fisher and Yates (11), and the logarithms from seven-figure tables.

The addition (+) leads to an upper limit, the subtraction (−) to a lower limit. The correction terms are always added, never subtracted.

To find, for instance, the upper limit ($P = 0.005$) for our sample:

$F\sqrt{Aq} = 2.5758\sqrt{Aq}$. Log $F = 0.4109$. Log $\sqrt{Aq} = 0.6606$ (see above). Therefore log $F\sqrt{Aq} = 1.0715$. Therefore $F\sqrt{Aq} = 11.79$.

From Table III the correction term for 4.783% (approximately 4.8%) = + 2.84.

Therefore $A + F\sqrt{Aq} +$ correction term $= 22.00 + 11.79 + 2.84 = 36.63$. This is the limit in terms of number of A's in a sample of 460.

Stage (4).—Convert this absolute value into a percentage, i.e., $36.63 \times 100/460 = 7.96\%$.

The reader will arrive at a very similar result by using Table II as in Example 10:

Under percentage of A's $= 5$, interpolate for $N = 460$ between 450 and 500. Under percentage of A's $= 3$, interpolate for $N = 460$ between 400 and 500. Interpolate for 4.78 between 3 and 5%.

Although Graphs 4, 5, and 6 (= Figs. 9, 10, and 11) are not specially designed to cater for samples of less than 1000, it will be seen that for the present example ($N = 460$; percentage in sample $= 4.8$ approximately) interpolation in Graph 4 between the lines for 5% and 3% indicates an upper limit of approximately 8%, as was found by calculation.

1. Argument from Sample to Population (*continued*)

(2) Samples with More than Two Classes

Example 13

Among 50 individuals (persons or animals) receiving a certain inoculation, or infected by a certain organism, the reaction is as follows: A (mild), 13; B (moderate), 17; C (severe), 12; D (very severe), 8. Are we justified in believing that these discrepancies are due to something more than chance, i.e., are they anything more than one should expect in a random sample of a population in which the true ratio is 1 : 1 : 1 : 1? In other words, is there an equal probability ($\frac{1}{4}$ or 0.25) for each of the four classes?

Method

To use a method such as gives the information for two-class samples in Tables IA, IB, and II would be laborious, and we substitute a method that, with proper precautions, is sufficiently accurate—the chi square (χ^2) test. The rationale of this test is discussed in Section C, Note 9. Here we are concerned with the method of using it.

The quantity chi square can be described in general terms as *a measure of the discrepancy between an observation and a hypothesis*. The greater the discrepancy, the larger is chi square, and, since the random sampling variations in chi square are well known, we can find in a table of chi square whether the discrepancy is greater than is usually attributed to chance. The hypothesis

in the present example is that the population has equal frequencies in the four classes, A, B, C, and D, i.e., that the ratio is $1:1:1:1$; but any other ratio, representing a hypothetical population composition, can be tested in the same way. For example, in a breeding experiment we know that, if a certain four characteristics are distributed among the progeny according to a certain Mendelian law, the animals should be distributed in four classes in the ratio $9:3:3:1$. There can, also, be any number of classes.

On the hypothesis of equal frequencies we should expect in Class A one-quarter of the total (50), i.e., 12.5 individuals. This is the 'expected' or 'hypothetical' or 'theoretical' value (t) corresponding to the 'observed' or 'actual' value (a), i.e., 13. Now find the difference between a and t (i.e., 0.5), square it, giving 0.25, and divide by t, to give 0.25/12.5, i.e., 0.02. (Two decimal places are usually enough, and a slide rule or a four-figure logarithm table lightens the work.)

Proceed in this way for all four classes:

Class	a	t	$(a - t)$	$(a - t)^2$	$(a - t)^2/t$
A	13	12.5	0.5	0.25	0.02
B	17	12.5	4.5	20.25	1.62
C	12	12.5	0.5	0.25	0.02
D	8	12.5	4.5	20.25	1.62
	50	50.0		Chi square	= 3.28

(The t values would, of course, differ in the different classes if the hypothetical ratio were not one of equality, e.g., if it were $9:3:3:1$.)

Turn now to Table VII. Two features in the table require comment: (1) Degrees of freedom, (2) Probabilities.

Degrees of Freedom

When a chi square value is calculated, as in the present example, by testing the frequencies in a sample against an exact population ratio, known or hypothetical, *the number of degrees of freedom is one less than the number of classes in the sample*, e.g., four classes give three degrees of freedom. This rule, without explanation, is sufficient in practice and a full explanation would be long and complicated.

Note, however, that with a total of four classes, when we have calculated the t values for any three (even if the t values are not equal, as they are in the present example) we know, from the total number of individuals, what the remaining t value must be, i.e., it is not independent or 'free'.

Probabilities of Chi Square

In Table VII, for three degrees of freedom, a chi square value of 7.815 has a probability, P, of 0.05. Our value of chi square, 3.28, is far below this; therefore there is *no significant difference* between our sample and the hypo-

thesis (a ratio of $1 : 1 : 1 : 1$). If the sample had given a chi square value greater than 7.815 (P less than 0.05) we should have said there was a *significant difference*, and if chi square had been greater than 11.345 ($P = 0.01$) we should have called the difference *highly significant* or *very significant*.

In terms of the present example the information given by Table VII means that, if there were a population in which the ratio of the numbers in the four classes was $1 : 1 : 1 : 1$, and if we took a large number of random samples of 50 and calculated chi square for each sample, only 0.05 (i.e., 5%) of the chi square values would be more than 7.815 and only 0.01 (i.e., 1%) of the chi square values would be more than 11.345. More exactly, we should approach closer to these proportions by taking more and more samples.

Comments

(1). There may appear to be a discrepancy between the levels of significance ($P = 0.05$ and 0.01) adopted in the use of chi square tables and the half-values (0.025 and 0.005) used for Tables IA, IB, and II; but the standards are really the same (see Section A3 and Section C, Note 11).

(2). Tests on samples containing more than two classes do not lead as directly to an estimate of confidence limits as with twofold classifications. When there are three or more classes the variety of possible population frequencies is enormous, and there is no one set of limits. We can, however, set up any hypothetical ratio and ascertain as above whether the sample agrees or disagrees with the hypothesis.

Precautions in the Use of Chi Square in Argument from Sample to Population

For reasons discussed in Section C, Note 10, certain precautions must be taken in the use of chi square, as in this example, to test sample frequencies:

(1). If t in one or more classes is less than 5, it is usually best to add those classes to neighboring classes. Thus, a five-class sample (four degrees of freedom) may have to become a three-class sample (two degrees of freedom), or even a two-class sample, suitable for testing by Tables IA, IB, or II. (For the exception to this rule see (3) below.)

(2). If t in each class is five or more, and especially if it is 10 or more, one can confidently accept a verdict of 'not significant', 'significant', or 'highly significant', but it is uncertain how far one can trust the chi square probabilities in greater detail.

(3). Even when t in one or more classes is less than 5 (down perhaps to 1) there need be little hesitation in accepting a verdict of 'nonsignificance' if the chi square value is well below the significant level, because in general the tendency for a low t value is to make the chi square probability lower than the true probability, i.e., to heighten the suggestion of significance.

Note.—Chi square can be used, as in this example, when there are only two classes in the sample (Section C, Note 11).

2. Comparison of Samples

(1) Two Samples, Each in Two Classes

Example 14

Twenty-four rats are subjected to a certain vapor that is liable to cause death. Two treatments, *A* and *B*, are applied, strictly at random, to equal numbers of animals.

	Died	Survived	Total
Treatment *A*	4	8	12
Treatment *B*	9	3	12
Total	13	11	24

Treatment *B* is already known to have some beneficial effect. Is there any evidence that Treatment *A* tends to be more successful? We do not know whether *A* may be better or worse than *B* or of the same value. Therefore we ask: Is there a significant difference between the frequencies of death in the two samples?

Method

For general principles see Section A4, and for further discussion see Section C, Note 12.

Because the samples are equal and neither contains more than 20 individuals, use Table IV, rearranging the samples thus:

	Survived	Died
Sample (1)—Treatment *B*	3	9
Sample (2)—Treatment *A*	8	4

This rearrangement is necessary because, to avoid duplication in Table IV, the sample with the more unequal proportions is called Sample (1)—here 3 : 9 rather than 8 : 4; and the smaller quantity in Sample (1) is always placed on the left. *Note.*—These figures represent actual frequencies (numbers of individuals) although, for typographical convenience, they are separated by a colon, as are ratios.

In Table IV find $N = 12$, and under that, in the column headed "Sample No. (1)" find 3 : 9. In the adjacent section of the column headed "Sample No. (2)" find 8 : 4, and read off the probability, 0.0498. Since this is greater than 0.025 the observed samples show no significant difference in frequency of death.

Interpretation

There is not sufficient evidence to indicate which treatment, if either, is the better.

Comments

(1). The misleading impression created by percentages can be illustrated by conversion of the data:

	Died	Survived
Treatment *A*	33.33%	66.67%
Treatment *B*	75.00%	25.00%

The difference, although 41.67%, has been shown to be nonsignificant. Other expressions are still more misleading, e.g., "Animals treated by *B* had two and a quarter times the death rate of the animals treated by *A*—an increase of 125%."

(2). Table IV was prepared because of the numerous small-scale experiments in which equal samples are used, and because in small samples the chi square method, used in later examples, is often too inaccurate. Exact probabilities for all possible combinations are given because it may be desired to extract as much information as possible from small samples—see Comment (6).

(3). Equality of samples is, of course, not necessary for the proper assessment of evidence, because all reliable tests give due weight to sample size, but equality is very desirable for other reasons (see Section A5).

(4). If the original samples had been unequal, but had given results as follows:

	Died	Survived	Total
Treatment *A*	4	9	13
Treatment *B*	8	3	11
Total	12	12	24

we could still have used Table IV because there would have emerged two equal samples (died and survived).

(5). Inspection of the smallest samples in Table IV shows that, with equal samples, we require four individuals in each before we can demonstrate a significant difference (P less than 0.025) and even then the samples must have directly opposed composition (0 : 4 and 4 : 0). Similarly, for a highly significant difference (P less than 0.005) we need two samples of five.

(6). In this example we have supposed that we did not know at the outset whether *A* or *B* might in reality be the more successful, if, indeed, there were any real difference. The same figures might, however, represent a different situation, because we might be quite sure that *A* could not be any *less* effective than *B*, or the sample labeled "Treatment *B*" might be untreated control animals, and we might be quite sure that Treatment *A* could do no harm. The question would then be: Is the death rate of treated animals significantly lower than the death rate of controls?

This situation, the 'one-sided comparison', was mentioned in Section A4 and is discussed in Section C, Note 5. In most instances the two types of situation are treated in these Examples as if they were the same. Thus, taking the original table of the present example, we have decided that there was no significant difference between the effects of A and B, because P was greater than 0.025. If the table had been in the form 'treatment versus no treatment', we should have proceeded in exactly the same way and concluded that there was no significant difference, and we should then have taken this as equivalent to saying that treatment was not significantly better than no treatment. If, however, the investigator desires to use other criteria with one-sided comparisons, e.g., $P = 0.05$ instead of 0.025, Table IV provides him with the necessary probabilities for equal samples up to $N = 20$.

Example 15

In Section A4 the following table was given:

Treatment	Recoveries	Deaths	Total
V	2	7	9
W	5	1	6
Total	7	8	15

Is there evidence of a real difference between the effects of the two treatments?

Method

Neither sample contains more than 20 individuals, but the samples are not equal. Therefore use Table V. In the column headed "Larger Sample" find $N_1 = 9$ (the V-treated sample) and in the adjacent column look for $N_2 = 6$ (the smaller sample, W-treated). If the frequencies in N_1 are 2 and 7, when the smaller sample ($N_2 = 6$) contrasts as strongly as possible (6 and 0) the difference is significant ($P = 0.0060$, i.e., less than 0.025) but is not highly significant; and a smaller difference, e.g., 5 and 1 as in our present problem, is not significant.

Interpretation

There is no evidence that V and W are different in respect of the frequency of deaths.

Comments on Table V

(1). Note that, to save duplication, the table has been arranged so that the smaller frequency in N_1 is on the left (2 : 7, not 7 : 2).

(2). Following down $N_1 = 9$ and $N_2 = 6$, note that frequencies of 3 : 6 and 6 : 0 are significantly different, but there is no entry for the next stage, i.e., 4 : 5 in N_1, because even when N_2 differs as much as possible from this, the difference is not significant.

(3). To appreciate more fully the structure of Table V, observe some other entries. Thus, under $N_1 = 15$ and $N_2 = 4$, the difference between the frequencies 2 : 13 and 4 : 0 is highly significant, but no other sample of four differs significantly from a sample of 15 when the frequencies in the latter are 2 and 13. Again, under $N_1 = 15$ and $N_2 = 14$, with frequencies of 7 and 8 in N_1, 14 : 0 shows a highly significant difference. Keeping the 7 : 8 constant and changing the frequencies in N_2 step by step we find that 13 : 1 shows a significant difference, but 12 : 2 does not, nor does 11 : 3, nor any of the other possible arrangements up to and including 2 : 12. A still greater change in the same direction, however, to 1 : 13, gives a significant difference, and the last move, to 0 : 14, takes us into the highly significant class. In terms of the present type of example:

Treatment	Recoveries	Deaths	Verdict
V	7	8	A significantly higher recovery rate with W
W	13	1	
V	7	8	No significant difference
W	2	12	
V	7	8	A significantly higher recovery rate with V
W	1	13	

Example 16

In an investigation (13) of the *in vitro* sensitivity of *Hemophilus influenzae* to penicillin, 18 strains of the organism were isolated from cerebrospinal fluid and inoculated on plates of blood agar containing 0.5 units of penicillin per cc. of the medium. The cultures were classified according to degree of growth and according to the characteristics of the strain, smooth or rough.

Strain	Growth		Total
	Heavy or moderate	Slight or nil	
Smooth	4	8	12
Rough	5	1	6
Total	9	9	18

The strains appear to differ in amount of growth. Is the difference significant?

Method

The two unequal samples (smooth and rough) can be tested by Table V as in Example 15. Under $N_1 = 12$, $N_2 = 6$, we find that, when the frequencies in N_1 are 4 and 8, the frequencies in N_2 must be at least 6 and 0 to show a significant difference, instead of 5 and 1 as here. Our sample shows no significant difference, i.e., P must be greater than 0.025. In this case,

however, we can find the exact probability by taking advantage of the equality of the two 'growth' samples (nine in each sample). Use Table IV as in Example 14. Under $N = 9$, for a frequency in N_1 of 1 : 8 and a frequency in N_2 of 5 : 4, $P = 0.0656$.

Example 17

In a report on an investigation of traumatic shock it was stated that, after dogs were experimentally injured by a certain technique, if they were kept at an environmental temperature of 95° F., 73% died, whereas if they were "cooled to an equivalent degree" only 40% died. The report did not state the actual number of animals and therefore the percentages were useless, but inquiry revealed that 15 dogs had been heated and 10 cooled, with the following results:

	Died	Survived	Total
Heated	11	4	15
Cooled	4	6	10
Total	15	10	25

Is there any evidence that heating and cooling differ in their effects on frequency of survival?

Method

In Table V look for the larger sample under $N_1 = 15$ and the smaller sample under $N_2 = 10$. When the frequencies in N_1 are 4 and 11, as here, and the frequencies in N_2 are 8 and 2, or 9 and 1 (or, of course, 10 and 0) the difference between the samples is significant, but if there is any greater similarity between the samples, e.g., 7 : 3 or 6 : 4 in N_2, the difference is not significant.

Interpretation

There is not sufficient proof of a real difference in effect between heating and cooling. So far as this sample shows, heating may tend to *lower* the frequency of death.

Example 18

In 20 patients with meningitis (31) due to *Hemophilus influenzae*, five were treated with sulphonamides alone, and only one recovered. Fifteen had combined sulphonamide–penicillin treatment and eight of them recovered. Examine the statement: "The total number of cases is too small and the methods of treatment varied too much to allow of statistical assessment. The only conclusion to be drawn is that the encouraging recovery rate suggests pushing to the limit the combined sulphonamide–penicillin treatment. . . . . ." The first step is to see how far chance could account for the apparent difference in the effects of the two treatments.

Method

Arrange in a fourfold table:

	Deaths	Recoveries	Total
Larger sample (sulphonamide–penicillin)	7	8	15
Smaller sample (sulphonamide alone)	4	1	5

In Table V, under $N_1 = 15$ and $N_2 = 5$, with frequencies in N_1 of 5 and 10, a sample of five with frequencies of 5 and 0 shows a significant difference; but no further entries are given, because, if the frequencies in N_1 are 6 : 9, 7 : 8, etc., a sample of five shows no significant difference even if its frequencies are 5 : 0.

Interpretation

Accepting the usual criteria of significance, we can assert that the samples show no reason why we should prefer the one treatment to the other.

It may be suggested, however, that in treating such a serious disease we should not demand as high odds (as low P values) as usual. If so, we must assess the probabilities exactly, by the method of Section C, Note 13. This is equivalent to providing an item for Table V in the region where our observed samples would fit, and P, thus determined, is 0.2214.

An investigator who is familiar with the principles of contingency tests (Section A4; Section C, Notes 12 and 13) will realize that this probability has the following implications:

(1). If he accepts the observed samples as indicating superiority of the combined treatment, and if he would have accepted, on the same basis, samples that appeared to favor the treatment by sulphonamide alone, he is accepting standards that will lead him to proclaim a real difference between treatments in more than 44% of his investigations in which there is no real difference (twice 22.14%).

(2). Even when he knows that one treatment (e.g., the combined treatment in this example) cannot be any worse than the other, if he accepts $P = 0.2214$ as indicating real superiority of one treatment he will proclaim superiority in more than 22% of his investigations in which there is no superiority.

Comments

This example deserves careful consideration because it illustrates one of the most vital problems of clinical research. Referring to the statement quoted at the beginning we can say:

(1). No sample is too small for statistical assessment.

(2). It appears to be felt that, in addition to the distinction between the two basic treatments (sulphonamide and sulphonamide–penicillin), there were so many differences in methods of treatment that the two samples would not be comparable. They would have been comparable, however, if the two basic treatments had been allocated strictly at random. Some of the ancillary factors would tend toward recovery, others toward death, and some patients

would have a predominance of recovery factors, others a predominance of lethal factors. By strict randomization these two classes of patient would have had an equal chance of appearing in the sulphonamide group and in the sulphonamide–penicillin group.

(3). Since the samples were not randomized they may have obscured some advantage or disadvantage in either of the treatments, and this would still be true even if our analysis had shown a significant superiority in one of them. As it is, our test has merely shown that there is not sufficient evidence to justify preference of either treatment.

(4). The physician might say: "I am unwilling to experiment with my patients. If, because of some suggestive work by other observers, or on the principle that two antibacterial agents are likely to be better than one, I feel that it would be better to give the combined treatment, I consider it my duty to do so." Therapeutically he would be correct, but he should then realize:

(*a*). That his procedure would thereby cease to be an investigation.

(*b*). That the "suggestive work by other observers" may have little sound evidence to support it—hence many of the changes in therapeutic fashions. If it were realized how often the apparent evidence in favor of some form of treatment could be explained by bad sampling or by the effects of chance, practitioners would probably not think that they were risking their patients by depriving them of such a treatment.

(5). Certain compromises might be suggested:

(*a*). To apply the sulphonamide treatment to the milder cases, the combined treatment to the more serious. But even if the combined treatment were better, unless it were very much better, one might easily emerge with a nonsignificant difference. Moreover, the experiment would probably break down when a mild case became more serious and the physician would feel impelled to apply the combined treatment.

(*b*). To give the combined treatment, but vary the amounts of penicillin and sulphonamide in different groups. Here again the experiment would be successful only if the plan were strictly adhered to.

(*c*). To increase the number treated by the combined treatment and compare the results with the five already treated by sulphonamide alone. But the sample of five and the sample treated by the combined method would not be strictly comparable because not random, and the same criticism applies to a comparison of data from one hospital (or physician) using sulphonamides alone with those of another using the combined treatment.

(6). Without experimentation no therapeutic advance can be made, and the practitioner is sometimes faced with the difficult question whether he shall apply a treatment that may entail some risk, or loss of some possible advantage, to a few patients in order to secure knowledge that may benefit a much larger number. One way of minimizing the risk is to plan the experiment well and to analyze the results in such a way as to extract the maximum amount of information from a small sample.

Example 19

Treatments *V* and *W* are tested on two equal samples—30 individuals in each.

Treatment	Deaths	Recoveries	Total
V	14	16	30
W	24	6	30
Total	38	22	60

Is there any evidence that the treatments differ in their effects?

Method

The common method of treating this would be by the method of Example 20; but, for equal samples, Table VI gives some useful indications of minimum differences, expressed in percentage form. The table appears somewhat complicated because, for any pair of samples (1) and (2), there is not just one minimum significant difference between the percentages of *A*'s in the samples. It varies according to the actual percentage of *A*'s in Sample (1), and it varies according to whether there are more *A*'s or fewer *A*'s in Sample (1) than in Sample (2).

To use the table, proceed as follows:

(1). Find the percentages of the two classes in the samples:

	Deaths	Recoveries	Total
Sample (1) *V*	46.67%	53.33%	100%
Sample (2) *W*	80.00%	20.00%	100%
Difference	33.33%	33.33%	

(2). Take either sample as Sample (1) and from it determine which class is to be *A*—the class that contains not more than 50% in Sample (1). If we take *V* as Sample (1), deaths (46.67%) will be *A*'s, recoveries will be not-*A*'s.

(3). Find *N* (here 30) in Table VI and, if, as here, there are more *A*'s in Sample (2) than in Sample (1) use the left half of the table.

(4). The percentage of *A*'s (46.67) lies between the tabulated values 25 and 50. To be highly significant the difference would have to be 36.67, instead of 33.33 as in our samples. To be significant it need only be 30.00%. The same conclusion is reached if we take the *W*'s as Sample (1), again using the left half of the table. (Note that the blanks in the right half indicate that maximum possible differences do not reach the specified level of significance.)

Interpretation

Our samples show a significant difference in death rate—a significant association between treatment and outcome.

Comments

. (1). Refer to Example 18. In the present example, V corresponds to the sulphonamide–penicillin treatment, W to the sulphonamide treatment. The percentage frequencies of deaths are the same, but in the present example N in each sample is 30. This shows that, if the proportions remained the same as in Example 18, we should require samples of almost 30 to prove a significant difference.

(2). Note some relations between N and the minimum significant difference in Table VI. If the percentage of A's in Sample (1) is zero, we can, at most levels in the table, halve the required difference by doubling N or we can reduce the difference to one-third by trebling N—a simple inverse relation. Where the percentage of A's is 50 there is approximately an inverse square-root relation, e.g., by quadrupling N we only halve the required difference. Even where the percentage is not 50, we can form a useful estimate of required sample size by assuming the inverse square-root relation (Example 36). The estimates will tend to be too high, but this is safer than the opposite error.

(3). For convenience of interpolation two columns from the left half of Table VI have been repeated in the right half.

Example 20

In an investigation (16) of the possible value of DDT as a preventive of scabies, soldiers with and without scabies were questioned regarding the use of DDT during the previous two months, either as a dusting powder or impregnated in their shirts.

	DDT	No DDT	Total
Soldiers with scabies	29	. 23	52
Soldiers without scabies	64	36	100
Total	93	59	152

Was there any evidence that DDT tended to prevent scabies?

Since we should not expect soldiers who used DDT to have a higher incidence of scabies than those who did not, this is really a one-sided comparison (Section A4, and Section C, Note 5), but we shall as usual test it for the significance of the difference between the two samples, as if DDT and some other agent were being compared and we had no knowledge which might be more effective.

Choice of Method

As the samples contain more than 20 individuals we cannot use Tables IV or V, and since they are unequal we cannot obtain any indication from Table VI. We therefore use chi square (χ^2) already described in Example 13 as a measure of the discrepancy between an observation and a hypothesis. The hypothesis here is the same as when Tables IV to VI are applied, i.e., that the samples are from the same population—in the present problem, that

there is no real difference in scabies incidence between those who use DDT and those who do not. In Example 13 chi square tested a sample against a hypothetical population ratio. Here we are using it with a contingency table and the arithmetical technique is different. In Section C, Note 14, the step-by-step technique is given, but a short method is easier.

Short Method of Calculating Chi Square for Fourfold Tables

Substituting letters for numbers in the fourfold table, we write:

a	b	$a + b$
c	d	$c + d$
$a + c$	$b + d$	$a + b + c + d = N$

For convenience we speak of a, b, c, and d as occupying the four *cells* of the table, and refer to the four *subtotals* (*marginal totals*) and the *grand total*.

We can now use the formula:

Chi square (corrected for continuity—see below) =

$$\frac{(ad \sim bc - N/2)^2 \times N}{(a + b)\,(c + d)\,(a + c)\,(b + d)}$$

where $\sim$ means 'difference between', regardless of $+$ or $-$ sign.
In words the procedure is:

(1). Cross multiply the contents of the cells to obtain two products.

(2). Find the difference between the products and reduce it by one-half the grand total. This reduction is the "correction for continuity" referred to in Section C, Note 10. Observe that it is a *numerical* reduction of the difference between the products, regardless of the sign, positive or negative, of that difference.

(3). Square the result (2) and multiply this square by the grand total.

(4). Divide the quantity found in (3) by the product of the subtotals.

Sufficiently precise results can be obtained by four-figure logarithms, often by a slide rule; but it is desirable to do the first pair of multiplications on the upper line by longhand.

Applying the formula to the present example, chi square (corrected for continuity), or $\chi_c^2 =$

$$\frac{(29 \times 36 \sim 64 \times 23 - 152/2)^2 \times 152}{52 \times 100 \times 93 \times 59}$$

$$= \frac{(1044 \sim 1472 - 76)^2 \times 152}{52 \times 100 \times 93 \times 59}$$

$$- \frac{(428 - 76)^2 \times 152}{52 \times 100 \times 93 \times 59}$$

$$\frac{352^2 \times 152}{5200 \times 93 \times 59} = 0.66$$

Turn now to Table VII. As in Example 13, two features in the table require comment: (1) degrees of freedom, (2) probabilities.

Degrees of Freedom

When a chi square value is found, as in the present example, by comparing two or more samples in a contingency table the rule is: number of degrees of freedom = (number of rows of cells *minus* one) × (number of columns of cells *minus* one). Rows and columns of totals are not counted. For a four-fold table, therefore, degrees of freedom = $(2 - 1) \times (2 - 1) = 1$. (For further remarks on the term 'freedom' see Section C, Note 14.)

Probabilities of Chi Square

In Table VII, for one degree of freedom a chi square of 3.841 has a probability, P, of 0.05. Our value of chi square (0.66) is very far below this.

Interpretation

There is no significant difference between the incidence of scabies in the two samples, i.e., no evidence that DDT, under the conditions specified, tended to reduce the incidence of scabies.

Comments

(1). As in Example 13, with chi square we use $P = 0.05$ and $P = 0.01$ as standards in assessing significance, instead of 0.025 and 0.005 used in Tables IV, V, and VI; but the standards are really the same (see Section C, Note 14), and $\frac{1}{2}P$ from chi square is an approximation to the P value that would be found by the exact method used to produce Tables IV, V, and VI.

(2). Chi square for this example is worked out by the step-by-step method in Section C, Note 14, but at present it is desirable to know one step—multiply together the two lowest subtotals and divide by the grand total, to give the minimum expected or theoretical value, m. In this example $m = 52 \times 59/152 = 20.2$. The meaning of the procedure is seen when the step-by-step method is used. It is introduced here because it enters into the rules for chi square, now to be given.

(3). Some investigators still use the standard deviation or standard error, $\sqrt{Npq}$, instead of chi square, for comparison of samples. This is not to be recommended (Section C, Note 15).

Precautions in the Use of Chi Square to Compare Two Two-class Samples (Fourfold Contingency Tables)

Tables IV, V, and VI were prepared because the approximation given by chi square ceases to be sufficiently close as the samples become smaller, and especially as m (the minimum expected value—see Comment (2) above) becomes smaller. For samples not covered by the tables, i.e., *larger than 20*, chi square will seldom lead one astray if the following rules are observed. (For the basis of the rules see Section C, Note 20.)

(1). When m is not greater than 1, find P by the exact method—see Section C, Note 13.

(2). When m is greater than 1, use chi square (corrected for continuity) to test for significance, using $P = 0.05$ (chi square $= 3.841$) as the boundary between significance and nonsignificance, and $P = 0.01$ (chi square $= 6.635$) as the boundary between significance and high significance; but unless m is more than 10 do not assess the probability from Table VII any more closely. The only serious doubt arises in borderline cases:

(a). If m is greater than 1 but not greater than 5, a chi square of 3.5 to 4.0 is of doubtful significance. Employ the exact method or increase the size of one or both samples.

(b). If m is between 5 and 20 a chi square of 3.7 to 3.9 is doubtful, but more likely to indicate significance than nonsignificance. As m increases above 20 the accuracy becomes greater.

(c). If m is not over 10, do not accept a verdict 'highly significant' unless chi square is over 7.

(3). With m over 10, and especially over 20, the P values of Table VII are reasonably safe, i.e., the $\frac{1}{2}P$ intervals can usually be accepted as indicating what would be found by the exact method. If there is an error it will usually make $\frac{1}{2}P$ somewhat greater than it ought to be.

Note.—Sometimes the correction for continuity reduces the quantity within the brackets of the chi square formula to zero or a negative quantity. This is because the correction tends to overcorrect. Calculation cannot continue but the verdict is 'not significant'.

Example 21

In an investigation (7) of the mode of spread of poliomyelitis the virus was sought for in the feces. One series showed:

	No. of households where a case of poliomyelitis occurred	No. of households having contacts with cases outside the home	Total
Virus discovered in one or more members of household	6	8	14
Virus not discovered	2	37	39
Total	8	45	53

Is there a significant association between degree of contact with cases and frequency of presence of the virus?

Method

The samples are too large for Tables IV and V. See *Precautions* in Example 20, and find m, the minimum expectation.

$m = 8 \times 14/53 = 112/53$—a little over 2. Therefore calculate chi square (corrected for continuity):

$$\frac{(6 \times 37 - 2 \times 8 - 53/2)^2 \times 53}{8 \times 45 \times 39 \times 14} = 8.7.$$

Since chi square is over 7, there is a highly significant difference between the samples.

Interpretation

There is strong evidence of an association between degree of contact and frequency of presence of the virus. This might mean either (*a*) that the occurrence of a case of poliomyelitis tended to spread the virus among members of the household, or (*b*) that poliomyelitis tended to occur more frequently when there was a more frequent presence of the virus in the feces of members of a household. It is not the function of an association test to prove causal relations.

Example 22

From the following data (12) one observer concluded that there was an association between tuberculosis and the condition of the sternum.

	Synostosis of sternum	No synostosis	Total
Persons with tuberculosis	4	7	11
Persons without tuberculosis	7	66	73
Total	11	73	84

Was the conclusion justified?

Method

The samples are too large for Tables IV and V. Use chi square (Example 20).

m is $11 \times 11/84$, i.e., between 1 and 2.

Chi square (corrected for continuity) =

$$\frac{(4 \times 66 - 7 \times 7 - 42)^2 \times 84}{11 \times 73 \times 73 \times 11} = 3.899 .$$

The precautions given in Example 20 show that this value, being between 3.5 and 4.0, is inconclusive. The exact method (Section C, Note 13) gives $P = 0.0336$—not significant.

Comment

Even if the association had been highly significant, the result would be almost meaningless without careful sampling methods. Structural and functional peculiarities often run in families and differ in frequency in different racial stocks, and so do tendencies to disease. Structural and functional changes occur with age and so do changes in the susceptibility to disease. Classification according to race and family, sex, and age are necessary. This would give a series of contingency tables, and the information, after each table was tested, might need to be combined—see Examples 30 and 31.

Example 23

An investigation (12) of 79 patients with diphtheria showed:

Antitoxin administration	Died	Recovered	Total
Not until 6th day	15	20	35
Before 6th day	3	41	44
Total	18	61	79

Test the statement that recovery tends to be more frequent with the earlier administration.

Method

The samples are too large for Tables IV and V.   Use chi square (Example 20).

m is $18 \times 35/79 = 8$ approximately.

Chi square (corrected for continuity) =

$$\frac{(15 \times 41 - 3 \times 20 - 79/2)^2}{18 \times 61 \times 35 \times 44} = 12.4 .$$

Chi square is far above 7, and therefore the difference is highly significant.

Comment

From other knowledge of diphtheria antitoxin we may readily accept it as the factor responsible for the very significant difference in frequency of recovery, but we should recognize that the data, as presented, do not justify this.   The chi square test has shown that the difference is much greater than we should expect chance to account for; but the test does not rule out bias in sampling, nor would a mere increase, however great, in the size of sample. Delay in one form of treatment (here antitoxin) may be merely part of a delay in proper care by physicians and nurses, and sometimes there is the added burden of a journey to be undertaken by the patient before treatment can be started.

Example 24

The comments on Example 6 pointed out that, if there were real justification for pairing of animals, the data of that example might refer to 10 pairs of animals from the same litter, the Treatments V and W having been allocated at random within each pair.   Let us suppose that the results were either recovery or death.   Specifying them in more detail we can write:

Pair No.	Result	Pair No.	Result
1	Both animals died	6	V recovered, W died
2	W recovered, V died	7	W recovered, V died
3	W recovered, V died	8	W recovered, V died
4	Both animals recovered	9	Both animals died
5	W recovered, V died	10	W recovered, V died

Of the pairs that show any difference, six show W better than V and one shows V better than W.

The hypothesis to be tested is that there is no detectable difference between the effects of V and W when applied to animals that are as closely alike as are animals in the same litter.   Example 6 showed that there was no significant difference from a 50% population frequency, P for this sample of seven being 0.0625.

It may now be asked: Cannot one take account of all 20 animals in a four-fold contingency table?

	R	D	Total
V	2	8	10
W	7	3	10
Total	9	11	20

To test the significance of the difference in this table means to test the hypothesis that the two samples, V and W, are, in respect of the frequencies of recoveries and deaths, random samples from the same population; but if the original pairing was justified the two samples would tend to be more alike than random samples, and a difference in the effects of V and W might well fail to demonstrate itself.

Table IV, under $N = 10$, shows that where frequencies in N_1 are 2 : 8 and in N_2 are 7 : 3, $P = 0.0349$—no significant difference. If P had been less than 0.025 we should have accepted it as evidence of the difference between V and W, even if the test applied to the pairs had shown no significant difference; but we should have concluded that the pairing of the animals had been unjustifiable. The contingency test should therefore be applied where the test of the results from pairs of animals has shown no significance; but this illustrates *the dangers of artificial pairing.* Unless there are very good reasons for pairing we run a serious risk of losing information that the same number of animals, unpaired, might have given. This information cannot be recovered by a subsequent contingency test, because, as already stated, we have artificially created samples that may be more alike than random samples.

Example 25

To explore the possibility that some forms of mental deficiency might be due to the *Rh* factor, mentally defective children were classified (28) as (a) mongols and those with well-established causes, e.g., cerebral palsy and birth trauma—the differentiated group, (b) those that could not be so differentiated. The mothers of these children were tested and showed:

Group	*Rh* negative	*Rh* positive	Total
(a)	6	47	53
(b)	14	42	56
Total	20	89	109

Is there a significant difference in the relative frequency of *Rh* negative mothers in the two groups?

Method

The samples each contain more than 20 individuals, and m, the minimum expected value, is $20 \times 53/109 = 9.7$ approximately. Chi square (corrected for continuity) =

$$\frac{(14 \times 47 - 6 \times 42 - 109/2)^2 \times 109}{20 \times 89 \times 53 \times 56} = 2.55 ,$$

which is well below 3.841, the value for $P = 0.05$. Since m is almost 10 we can accept the $\frac{1}{2}P$ intervals in Table VII as indicating the values of P that would be obtained by the exact method (Section C, Note 13) used to produce Tables IV, V, and VI. Here $\frac{1}{2}P$ is between 0.10 and 0.05, nearer the latter.

Interpretation

There is no significant difference in frequency of *Rh* negative mothers in the two groups.

Comment

The observations that prompted this investigation were some clinical observations and some discoveries in autopsies. These suggested that *Rh* incompatibility, if it did not cause death or marked physical defects, might cause mental defect. Therefore the investigators might wish to continue this investigation despite the fact that their results so far had not reached the conventional standard of significance. It is desirable in all such cases, however, to assess the existing evidence more precisely, and P found by the exact method is 0.0542, which agrees, as it should, with the $\frac{1}{2}P$ value from chi square already obtained, but it enables the investigator to estimate his possible error more precisely, and to decide whether it is worth while pursuing the investigation further—see Section C, Note 13.

Note.—Some investigators still compare samples by the standard error or standard deviation, $\sqrt{Npq}$, but this is not to be recommended—see Section C, Note 15.

Example 26

In the investigation used for Example 25, the following comparison was made. In the group classified as (*b*) in that example there were 14 *Rh* negative mothers out of 56, i.e., 25%. Observations by other workers were quoted to show that the proportion of *Rh* negative individuals in the general white population was approximately 15%. The question was asked: Does the sample of 56 with 14 *Rh* negative show a significant difference from the population value? The treatment of this question illustrates three common risks:

(1). The risk of accepting other observers' populations as equivalent to one's own.

(2). The risk of applying tests that give only approximate answers.

(3). The risk of accepting as population values data that are really estimates from samples.

Other Observers' Populations

The 15% frequency of *Rh* negatives was stated to be derived from random samples of the white population; but in such cases 'random' commonly means 'unselected', i.e., obtained without purposive sampling from the people or material available in the location where the observer was at work. But so many structural and functional features in human beings are apt to differ in different parts of the same country, and even in different regions of the same city, that, unless such variations have been thoroughly investigated, there is always a risk of bias. With regard to the *Rh* factor, marked differences between whites and other racial stocks have been recorded (19), and, even without further information, one should recognize the possibility of differences within different stocks of the so-called 'white' race. To minimize the risk of such bias one must choose for comparison samples within the same region.

The Risks of Approximations

The difference between the sample value (25%) and the population value (15%) was tested by a method commonly used, the standard deviation (Section C, Note 8) and pronounced significant; but Table IB gives a more accurate verdict. Number of A's in sample = 14; $N = 56$. The lower limit (for $P = 0.025$) is about halfway between 14.7% ($N = 55$) and 14.1% ($N = 57$), i.e., the sample is nearly, but not quite, significantly different from a population value of 15%. In many problems the distinction may be unimportant, but in assessing available evidence before setting up an elaborate investigation it is desirable to make the assessment as accurate as possible.

The discrepancies introduced by methods of approximation become greater with smaller samples and lower percentages. Indeed it may often be desirable to obtain even greater precision than that of Tables I and II, by the binomial expansion, especially where one-sided comparisons are being made—for method see Section C, Note 18; for application to the present problem see Section C, Note 8—Example (2).

The Risk of Accepting Estimates as True Population Values

The paper from which the 15% frequency of *Rh* negative individuals was quoted showed that it was an estimate—the frequency found in a sample of 334 persons. In this particular problem some other samples had indicated a similar frequency; but often percentage frequencies are quoted, in textbooks or articles, without indication of sample size (which may be small) and then are used by other workers as if they were true population frequencies. For the effects of such methods on the present problem see Section C, Note 19.

2. COMPARISON OF SAMPLES (*continued*)

(2) MORE THAN TWO SAMPLES; MORE THAN TWO CLASSES

Example 27

One hundred and twenty-nine men had been found susceptible to motion sickness induced by a swing. To each of five groups of these men drug mixtures of the same general type were administered, the difference between the groups

being in the detailed composition of the mixtures and in the interval between the administration of the drug and the subsequent test in the swing. (Percentages of men sick and not sick for each treatment are inserted in parentheses but are not to be used in the test.)

Treatment	No. of men not sick	No. of men sick	Total
V	13 (65.0%)	7 (35.0%)	20
W	7 (43.7%)	9 (56.3%)	16
X	24 (61.5%)	15 (38.5%)	39
Y	19 (67.8%)	9 (32.2%)	28
Z	20 (76.9%)	6 (23.1%)	26
Total	83	46	129

What is the evidence that the treatments differ in their effects?

Method

The data form a 2 × 5 contingency table, and chi square, *not corrected for continuity*, is to be used. The hypothesis to be tested (Section C, Notes 12 and 14) is that the five samples come from the same population with a ratio not-sick to sick as in the subtotals—83 : 46. On this hypothesis, estimate the hypothetical, expected, or theoretical value (t) corresponding to each of the 10 actual values (a) in the table. Thus, for V—not sick, $a = 13$, $t = 20 \times 83/129 = 12.87$. Similarly, multiplying in succession 16, 39, 28, and 26 by 83/129, i.e., 0.6434, we find the remaining t values for not-sick. For the sick we multiply the same totals by 46/129, i.e., 0.3566, and check the results by subtracting the t (not sick) values from the respective totals.

Treatment	Not sick				Sick			
	a	t	$a-t$	$(a-t)^2/t$	a	t	$a-t$	$(a-t)^2/t$
V	13	12.87	0.13	0.00	7	7.13	0.13	0.00
W	7	10.29	3.29	1.05	9	5.71	3.20	1.90
X	24	25.09	1.09	0.05	15	13.91	1.11	0.09
Y	19	18.02	0.98	0.05	9	9.98	0.98	0.10
Z	20	16.73	3.27	0.64	6	9.27	3.27	1.15
Total	83	83.00		1.79	46	46.00		3.24

(Note that the not-sick and sick must both be included in the calculation.)

Chi square = 1.79 + 3.24 = 5.03.

To enter Table VII, find the number of degrees of freedom for a 5 × 2 contingency table, i.e. (number of columns *minus* 1) × (number of rows *minus* 1)

$= (2 - 1) \times (5 - 1) = 4$. Table VII shows that our value of chi square is far below the value, 9.488, required for significance (at $P = 0.05$).

Interpretation

There is no proof that the treatments differ from each other in their effects. Therefore the mere fact that Treatment Z seems to give the best results is no reason for confining further attention to that treatment. There is no adequate reason for supposing it to be better than any of the others, nor is there adequate reason for believing that Treatment W is worse than any of the others.

Comments

(1). An investigator, noting that Treatment W seems to differ greatly from Treatment Z in its effect, might separate these from the rest and analyze the data in a fourfold contingency table—7 : 9/20 : 6. Even if he thereby found a 'significant' difference the result would be fallacious because the samples so selected are not random samples. They have been selected *on account of their differences in frequency*, and if one purposely selects samples from two widely separate parts of the same population (or frequency distribution) one will frequently find greater differences than we attribute to *random sampling* in tests of significance.

(2). The method of calculating chi square shown above is used for contingency tables with any number of rows or columns.

Precautions in the Use of Chi Square for Contingency Tables Larger than Fourfold

As stated with reference to fourfold contingency tables in Example 20, chi square gives an approximation to the exact results that would be obtained by much more complicated methods. It will seldom lead one astray with tables larger than fourfold if the following precautions are taken:

(1). Unless all t values are 10 or over, do not accept the P from chi square as a close approximation to the true value.

(2). If t in one or more cells is between 5 and 10, accept the verdict as indicating merely significance or nonsignificance.

(3). If t in one or more cells is less than 5, it is probably safe to accept a verdict of nonsignificance, because the tendency with low figures appears to be an exaggeration of the chi square, i.e., a lowering of P. For greater safety, or *if chi square indicates significance*, combine adjacent rows (or columns, or both) in such a way as to produce no t that is less than 5—see Example 28.

Example 28

Patients with serpiginous ulcer of the cornea were treated with prontosil and the visual acuity of their affected eyes, after discharge from hospital, was compared with the visual acuity in patients who, previous to the introduction of prontosil, had been treated by other methods—argyrol or mercurochrome (22). The data form a 2 × 5 contingency table:

acuity	Number of patients		Total
	Prontosil series	Previous series	
A 6/6 or better	6	1	7
B 6/6 to 6/18	7	8	15
C 6/18 to 6/60	5	3	8
D Less than 6/60	2	6	8
E Eye lost	0	2	2
Total	20	20	40

Was there a significant difference between the results in the two series?

Since the series were not random samples (see Comments below), all that we can do is to find out how far chance could account for the differences in the results.

Method

Apply chi square as in Example 27. In the first row, in place of the actual value 6, the theoretical value (t) is $7 \times 20/40$, i.e., 3.5, and similarly the other t values are found:

	Prontosil series	Previous series
A	3.5	3.5
B	7.5	7.5
C	4	4
D	4	4
E	1	1

Chi square = 8.14. Degrees of freedom = $(2 - 1) \times (5 - 1) = 4$.

P from Table VII is greater than 0.05, indicating a nonsignificant difference. However, t in most of the cells is less than 5; therefore for safety we pool adjacent cells in the contingency table. Sometimes the pooling of only two rows (or columns) is sufficient, but here, looking at the t values, we find it necessary to combine so many rows that the result is a fourfold table.

The following four variants can be made:

Visual acuity	Prontosil series	Previous series	Total
(1)			
6/18 to 6/6 or better	13	9	22
6/18 to zero	7	11	18
Total	20	20	40
(2)			
6/60 to 6/6 or better	18	12	30
Less than 6/60	2	8	10
Total	20	20	40

Visual acuity	Prontosil series	Previous series	Total
(3)			
6/6 or better	6	1	7
6/6 to zero	14	19	33
Total	20	20	40
(4)			
Still present	20	18	38
Eye lost	0	2	2
Total	20	20	40

If these tables had been larger than fourfold we should have tested them by chi square, and similarly if the samples had been larger than here. If chi square were to be used for these tables we should reject Variants (3) and (4) because they would contain t values lower than 5, and Variant (1) would be preferable to Variant (2) because the latter would still contain t values of 5 in the lower line. (If none of the t values in either variant were less than 10 it would be legitimate to adopt the one, namely, (2), that brings out the greater contrast between the two series.)

In the present case, with samples of 20, we can use Table IV to test all four tables. Under $N = 20$, we find P values for the four variants as follows:

Table	P
(1) 7 : 13/11 : 9	0.1703
(2) 2 : 18/8 : 12	0.0324
(3) 1 : 19/6 : 14	0.0457
(4) 0 : 20/2 : 18	0.2436

These results agree with the test applied to the original 2 × 5 table in showing no significance.

Interpretation

There is no adequate suggestion that the two forms of treatment differed in their effects.

Comments

(1). The pooling of two treatments, argyrol and mercurochrome, does not allow one to test prontosil against each of them.

(2). The example illustrates a common method of comparing clinical treatments. A new treatment is applied to all patients and the results are compared with a treatment previously applied to all patients. This is not equivalent to taking random samples. The two series may differ more than would random samples in many factors that will, or may, influence recovery, e.g., age incidence, sex, strain of microorganisms, differences in adjuvant treatment and nursing, differences in severity in hospitalized patients depending on availability of hospital space. Some of these, e.g., age and sex, can be

tested for, but for others we have to depend on such untested statements as: "There seemed to be no evidence that the severity of the disease differed in the two series." That this is not academic hair-splitting has been shown when clinical observers, comparing two treatments, have agreed to adopt strict random sampling in the comparison of the 'old' and the 'new', and have found that, if they had judged the effect of the 'old' treatment by their previous records they would have come to the opposite conclusion from the one reached by random sampling methods.

In the present example incorrect sampling may have obscured a real difference, in favor of one or other treatment, and this would still be true even if the test had shown a significant difference between the observed samples.

Example 29

Data (12) on sex ratios of stillborn children:

Group	No. of cases	No. of males per 100 females
I Nine months and older	31114	126
II Ninth month	8300	120
III Eighth month	8395	114
IV Seventh month	4967	125
V Younger than sixth month	2056	125

The data suggest a tendency towards a lower preponderance of boys at the eighth month than at other periods. Test the evidence.

Method

Sex ratios, as here, are, if anything, more misleading in appearance than are percentages, and they are not easily amenable to statistical tests. Therefore compute exact frequencies to the nearest whole number. For example, in Group I if there were a total (males and females) of 226 there would be 126 males; therefore the number of males is (126/226) of 31114 = 17347.

Arrange the results in a contingency table:

Group	Males	Females	Total
I	17347	13767	31114
II	4527	3773	8300
III	4472	3923	8395
IV	2759	2208	4967
V	1142	914	2056
Total	30247	24585	54832

Calculate chi square as in Example 27. Formulae (Fisher (9), Section 21) reduce some of the labor in analysis of tables such as this, in which one division is into only two classes (here males and females), but they are designed for calculating machines.

Done step by step as in Example 27, on a calculating machine, the following values for $(a - t)^2/t$ were found—the 10 contributions to chi square:

	Males	Females
I	1.9629	2.4150
II	0.5802	0.7138
III	5.4550	6.7113
IV	0.1324	0.1630
V	0.0543	0.0668

Their total, chi square, is 18.2547. Calculation by four-figure logarithms resulted in some discrepancies, especially in both columns of Group I, where the numbers involved were largest, but the discrepancies cancelled each other, giving a chi square of 18.25.

Enter Table VII with degrees of freedom $= (5 - 1) \times (2 - 1) = 4$. The chi square is beyond even the value for $P = 0.01$—a highly significant difference.

Interpretation

The sample strongly indicates that the population of stillbirths that it represents was not homogeneous in respect of male: female sex ratio throughout the months. The individual contributions to chi square show that the nonhomogeneity is mainly due to Group III—children at the eighth month.

Comment

The significant difference justifies a further search into the original data for possible causes. Before doing so one would apply further tests for homogeneity. By removing Group III and placing the remaining groups in a 4×2 contingency table one could again test by chi square. By fourfold tables one could compare Groups I and II, II and III, III and IV, IV and V.

3. COMBINATION OF INFORMATION FROM TWO OR MORE SAMPLES

In order to provide information on a certain question an investigator often performs a number of separate experiments, sometimes with a somewhat different procedure, or different materials, in each experiment. Sometimes no single experiment provides a conclusive (statistically significant) result, and sometimes some of the experiments do so and others do not. All the experiments, however, may seem to point to the same conclusion, and the experimenter wishes to combine the information.

The same type of experience is met by investigators who, owing to the nature of their material, must observe and analyze without experimenting. The question that confronts any such experimenter or observer is: Should he (1) combine the data, i.e., pool all his samples and apply a statistical test to the pooled data, or should he (2) combine the results, such as probabilities, previously obtained by testing each sample separately? If he adopts the second method another question arises: how should be combine the probabilities?

The common method of combining information is to pool the samples, but this, as will be .shown, entails a risk of fallacious conclusions from heterogeneous data.

Example 30—Combination of Data

In two groups of dogs all the animals were subjected to a treatment that is liable to cause death.   In each group half the animals, selected at random, were treated by methods that, it was thought, might prevent death;   the others were used as untreated controls.   The two treatments differed, but possessed a common factor.   The results were:

	Died	Survived	Total
Group *A*			
Treated animals	4	8	12
Control animals	8	4	12
Total	12	12	24
Group *B*			
Treated animals	3	8	11
Control animals	8	3	11
Total	11	11	22

How strong is the evidence that treatment tended to reduce mortality, either in the individual groups or in the series as a whole?   (Note.—Although the marginal totals for the columns (died and survived) happened to correspond numerically to sample sizes, this has no bearing on the problem.)

The two groups were not formed by taking 46 dogs and allocating them at random to the groups.   The experiments were independent in the sense that, after the observations in Group *A* were made, a second experiment, on Group *B*, was planned.   The animals might, indeed, not all be dogs, but dogs (*A*) and cats (*B*), or if all were dogs, they might be females (*A*) and males (*B*), or they might be immature males (*A*) and mature males (*B*).   The same method could apply if we had any number of groups and any number of classes within the groups.

Method

First find whether there is, within each individual group, significant evidence of the effect of treatment.

Using Table IV because the samples are equal and do not contain more than 20 animals in each, we find—

Group *A*: $N = 12$; $P = 0.1102$;   Group *B*: $N = 11$; $P = 0.0431$. Taking $P = 0.025$ as the standard for significance, we decide that neither group shows a significant evidence of the effect of treatment.*   The observed

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* *Although this is a one-sided comparison (Section A4) we are, as usual, applying the test as for two-sided comparisons.   Note that, even if we decided to accept Group B as showing significant evidence in favor of treatment, we might wish to strengthen the evidence by combining the information as is done here.*

difference in both groups, however, is a lower mortality among treated animals, and it is possible that by combining the information from both groups we might find convincing evidence that treatment had an effect. By pooling the two treatment samples and pooling the two control samples we could obtain a fourfold contingency table—two samples with 23 animals in each; but before doing so we must see whether we are thereby pooling samples that are unlikely to be random samples from the same population in respect of mortality; i.e., *we must test the two treatment samples for homogeneity and then test the two control samples*.

Group	Treated animals		Total
	Died	Survived	
A	4	8	12
B	3	8	11
Total	7	16	23

There is obviously no significant difference, but if there were doubt we should use Table V. If the samples were too large for Tables IV or V, or if there were more than two classes or samples, we should use chi square. Similarly the control samples show no significant difference.

Group	Control animals		Total
	Died	Survived	
A	8	4	12
B	8	3	11
Total	16	7	23

We can therefore combine the two treatment samples as if they were samples from the same population, and we can similarly combine the two control samples, to give a fourfold table:

	Died	Survived	Total
Treated animals	7	16	23
Control animals	16	7	23
Total	23	23	46

Chi square (corrected for continuity) $= 5.56$.

This is far above 3.841; therefore there is a significant difference in mortality between treated and control animals.

Interpretation

There is good reason to believe that the treatment lowers the mortality. From the previous tests of homogeneity we have no reason to believe that Treatment *A* differs from *B* in this respect, or that the two groups of animals differ in response.

Example 31—Combination of Probabilities and of Chi Square Values

To show how the information has to be combined when samples are not homogeneous, we take the following data from an experiment like that of Example 30.

	Died	Survived	Total
Group *A*			
Treated animals	0	12	12
Control animals	2	10	12
Total	2	22	24
Group *B*			
Treated animals	3	8	11
Control animals	8	3	11
Total	11	11	22

Method

Again it appears that the treatment tends to reduce mortality, but in neither group, with $P = 0.025$ as the standard, is the difference significant (Table IV).   Group *A*: $N = 12$; $P = 0.2391$.   Group *B*: $N = 11$; $P = 0.0431$.

Before combining the information we test for homogeneity in two contingency tables:

	Died	Survived	Total
Treated animals			
A	0	12	12
B	3	8	11
Control animals			
A	2	10	12
B	8	3	11

With $P = 0.025$ as standard, Table V shows lack of homogeneity in the controls, although not in the treated animals.

Treated animals: $N_1 = 12$, $N_2 = 11$—not significant;

Control animals: $N_1 = 12$, $N_2 = 11$—significant ($P = 0.0101$).

By pooling the two groups of controls we should be combining two samples that we have good reason to believe represent different populations.   We must therefore combine the information in a way that will avoid fallacious

inference. Two methods are available: (1) summation of chi square values; (2) conversion of probabilities into chi square values and summation of those values.

Summation of Chi Squares

If we add together any number of chi square values the result is a chi square value, and its significance can be found in a chi square table such as Table VII. To find the degrees of freedom we merely add together the degrees of freedom of all the contributing .chi square values. Since most contingency tests are performed by chi square we shall show this method first, although in this example it necessitates the calculation of chi square for the two contingency tables.

Group	Chi square	Degrees of freedom
A	0.5455	1
B	2.9091	1
Sum	3.4546	2

Table VII shows that this is below the level of significance ($P = 0.05$) and Fisher's table of chi square shows by interpolation that $P = 0.178$. (For method of interpolation see Fisher (9), Section 21.1.)

Conversion of Probabilities into Chi Square Values

This is very useful because it can be applied to any set of probabilities that have been obtained from independent tests of significance, e.g., in mensuration data. The method (Fisher (9), Section 21.1) is to take the natural (Napierian) logarithm of each probability, change its sign, and double it. This gives the corresponding chi square for two degrees of freedom. We then add together all the chi square values so obtained.

The simplest way to find the natural logarithms is to multiply the common logarithms by 2.303, and the multiplication can be postponed until after the summation.

Applying the method to the present example, we note that the probabilities obtained for Group A (0.2391) and Group B (0.0431) correspond to $\frac{1}{2}P$ values for chi square (see Section C, Note 14); therefore it is convenient here to double them (0.4782 and 0.0862) because we are going to use a chi square table for testing significance.

Group	P	Common logarithm of P	Degrees of freedom
A	0.4782	$\bar{1}.6796 = -0.3204$	2
B	0.0862	$\bar{2}.9355 = -1.0645$	2
		Sum $= -1.3849$	4

Sum of natural logarithms $= -1.3849 \times 2.303 = -3.1894$.

Sum with changed sign $= 3.1894$.

Sum $\times 2 = 6.3788 =$ chi square for four degrees of freedom.

Table VII shows that this is below the level of significance.   Fisher's table of chi square shows by interpolation that $P = 0.172$, very close to the value found by summating chi square directly.

Interpretation

When allowance is made for the heterogeneity of the material examined, there is not sufficient evidence that either treatment lowers the mortality.

Comments

(1). If the P value obtained by these methods (summation of chi squares, directly or after conversion of probabilities) had been less than 0.05, and if Treatments A and B had been the same, we should have concluded that treatment tended to lower the mortality.   We should not have known whether this was true for both groups considered individually, but until further evidence was obtained we should have been justified in assuming that it did—that, if immediate action were required, we could act on that assumption. If, however, the treatment had differed but possessed a common element, we should not have known how far the common element was responsible and how far each treatment had produced its effects by some feature appropriate to the group on which it was used.   The result would, however, have justified further investigation.

(2). For contrast with the above results, which showed no significant difference, the original samples were pooled, giving a treatment sample of 23 and an equal control sample.   The fourfold contingency table gave a chi square of 3.86, just beyond the significant value.   Since the control samples have been shown to lack homogeneity, it is unsafe to accept this result.

(3). When chi square values from fourfold tables are to be summated as in this example, it is stated (5) that they ought to be calculated without correction for continuity; but the question seems to require further investigation.   In the present instance the corrected values were used and, as has been shown, the results agreed closely with the results of combining the exact probabilities. The risk in using the corrected values is that the probability of the final result will be higher than it should be, but this is generally a less serious fault than claiming significance where it does not exist.

4. Confidence Limits for Differences Between Samples

If the difference between two samples is not significant there may, nevertheless, be a 'real' difference, i.e., a difference between the populations from which the samples were drawn.   For instance, two treatments are often compared and pronounced 'equally efficacious' although all that has been proved is that the experiment showed no significant difference, i.e., no proof that they were not 'equally efficacious'.   Larger samples might give convincing evidence that there was a real difference, but before enlarging his samples the observer would naturally say: "Is the real difference, if such exists, unlikely to be

more than, say, 5% greater success with Treatment A than with Treatment B? If not, it does not interest me."

Even if a difference between samples has been proved significant, a similar question arises, because the real difference may be either greater or less than the difference between the observed samples.

Example 32

The data of Example 20 are:

	Users of DDT	Nonusers of DDT	Total
Soldiers with scabies	29	23	52
Soldiers without scabies	64	36	100
Total	93	59	152

Chi square $= 0.66$—no significant difference.

The samples show, however, that of the 59 soldiers who had not used DDT, 39.0% developed scabies, as against 31.2% of the 93 who had used it—a difference of 7.8%. If there is a real difference in scabies incidence between users of DDT and nonusers, what is likely to be the maximum limit of the difference?

Method

The same procedure can be followed whether the samples have shown a significant difference or not. In the present instance, where there was no significant difference, assume that DDT actually tends to lower the incidence of scabies. For the sample of 93 users of DDT (31.2% scabies) find the *lower* confidence limit at $P = 0.025$. Since the number of A's (29 with scabies) is greater than 20, use Table II or Graph 2 (= Fig. 7). For $N = 93$, the required limit is approximately 22%. (For method of interpolation see Example 10.)

For the sample of 59 nonusers of DDT (39.0% scabies) find likewise the *upper* confidence limit at $P = 0.025$—approximately 52.5%. The difference between 22 and 52.5 is 30.5%.

To obtain wider limits and therefore higher confidence, we can use the $\frac{1}{2}$% levels ($P = 0.005$).

For the rationale of the method and the reliability of the estimates, see Section C, Note 16.

5. SIZES OF SAMPLES REQUIRED

At the beginning of any investigation, or certainly in its early stages, it is desirable to estimate the number of observations (size of sample) that would probably be required to establish some particular result with a specified degree

of precision. Sometimes, indeed, if investigators had, before starting their projects, made such estimates, based on information already known, they would have seen that they could not, with the material, facilities, time, and money available, arrive at any conclusive result.

Estimation of sample size can be considered in three types of problem:

(1). Argument from a sample to a population—Examples 33 and 34.

(2). Comparison of samples on the assumption that there is a difference between their populations—Example 35.

(3). Comparison of samples on the assumption that they come from the same population—Example 36.

Example 33

A certain blood substitute, transfused into 80 patients, produced no unfavorable reactions, but it was shown in Example 7, by use of Table IA, that in any population, represented by this sample, we should not trust the percentage of unfavorably reacting persons to be less than 6.4. Assuming that we found no unfavorable reactions when we used the substitute on more and more people, how many people should we examine before we could conclude that the percentage of unfavorable reactions was very unlikely ($P = 0.005$) to be more than 1 in 500, i.e., 0.2%?

Method

Table IA, under "Number of A's in sample $= 0$", shows that, if $N = 1000$, the upper limit ($P = 0.005$) is 0.53%. With these higher values of N the confidence limit is inversely proportional to N, e.g., at $N = 1000$ the value (0.53) is approximately one-half the value (1.1) at $N = 500$. At $N = 2000$ the value would be approximately one-half of 0.53, i.e., 0.265%.

To find N for the limit 0.2%, divide the value for $N = 1000$ by $N/1000$, i.e.,

$$\frac{0.53}{N/1000} = 0.2.$$

Therefore $530/N = 0.2$. Therefore $N = 2650$.

Interpretation

If we continued to find no unfavorable reactions we should require to investigate about 2650 persons before being justified in stating that the percentage of unfavorable reactions was very unlikely to be more than 0.2% or 1 in 500.

Example 34

In Example 1, Variant (3), vaccination against a certain disease had protected 15 rats out of 25 (60%), but it was shown that there was no adequate reason to suppose that it would protect more than 38.7% of such rats. Assuming that the investigator examined more rats and still found 60% protected, how many would he require to examine before he could feel reasonably confident ($P = 0.025$) that the protection rate was no less than 50%?

Method

In Table II find "Percentage of A's in sample = 40", i.e., the percentage unprotected. Find a value of N such that, when 40% of that sample are unprotected, one can say that it is unlikely that the true percentage unprotected is greater than 50%.

At $N = 100$ the upper limit ($P = 0.025$) is 50.2; at $N = 110$ it is 49.7. Therefore the required number of rats is between 100 and 110. For greater confidence ($P = 0.005$) one would require approximately 170 rats.

Comment

In an actual investigation, of course, the larger sample might show a higher or lower percentage than did the original sample, and the size of sample required would depend on that percentage. We could therefore assume various values for this percentage (subject to the proviso that the large sample did not differ significantly from the original sample), and use Table II to give estimates of necessary sample sizes. For most purposes, however, the one simple procedure, shown above, gives an adequate 'most likely' estimate.

Example 35

Example 20 presents the following data:

	Users of DDT	Nonusers of DDT	Total
Soldiers with scabies	29	23	52
Soldiers without scabies	64	36	100
Total	93	59	152

The incidence of scabies in users of DDT was 31.2%; in nonusers, 39.0%. Difference = 7.8%; but chi square (0.66) showed that this difference was not significant. If, however, the difference persisted when we examined more soldiers, we should reach a stage when we should pronounce it significant. How many soldiers should we require for this?

Method

Let it be supposed that we can investigate equal numbers of soldiers who have, and who have not, used DDT, and let N be the number required in each sample. In Table VI call the users of DDT Sample (1)—31.2% scabies; and call the nonusers Sample (2)—39.0%. There are more A's in Sample (2); therefore use the left half of the table. Locate 31.2% of A's in Sample (1) between 25 and 50%, and for the lower degree of confidence ($P = 0.025$) use the lower part of the table.

For $N = 200$ the minimum significant difference is between 9.50 and 10.50—about 10%; for $N = 500$ it is between 5.80 and 6.40—about 6%. The observed difference is 7.8%. Therefore the required N is roughly half-way between 200 and 500—say 350, i.e., a total of 700 soldiers.

To obtain a closer approximation we can now construct a table, with proportions (scabies: no scabies) as in the original samples, but containing 350 in each sample.

	Users of DDT	Nonusers of DDT	Total
With scabies	109	137	246
Without scabies	241	213	454
Total	350	350	700

Chi square (corrected for continuity) = 4.5 approximately. Therefore to provide a difference that is just significant (chi square = 3.841) we require fewer than 350 in each sample. If the samples are reduced to 300 each, chi square becomes approximately 3.5. Two samples with 320 in each give a chi square of approximately 3.9.

Another method commonly used for estimating sample sizes in such problems is based on the important fact that *chi square is proportional to the sizes of samples*, i.e., if we multiply the sample sizes and cell contents in a contingency table by any number, k, we multiply the value of chi square by k also. Thus, if we double the information we double the chi square value. Strictly, this relationship applies only to chi square values calculated without correction for continuity, but it is accurate enough for the first stage in estimates based on the corrected values of chi square. With such values it leads to an overestimate of the required sample size.

In the present example chi square (corrected for continuity) for the samples of total 152 is 0.66. For a significant difference we require a chi square of at least 3.841 (Table VII), i.e., nearly six times the original value. Trial with various factors, however, shows that it is sufficient to multiply the original samples by 4.4, giving a total of 670, in order to obtain a chi square (corrected) of approximately 3.8. Note that the total is greater than was required (640) when the samples were equal, because equal samples give more information than unequal samples of the same total size.

Finally, it may be undesirable or impossible to add to one of the samples, and the other must be increased greatly to compensate. The simplest method is to estimate roughly the required total as if both samples were equal in size, then transfer the increase to one of the samples and test by chi square. If the increase is too small (or too great), increase (or decrease) the one sample by 50 or 100, and test again. In the present instance one could start by increasing the DDT users from 93 to, say, 700, making the number with scabies 31.2% of 700, i.e., 218.

Example 36

In Example 17 there was shown to be no significant association between temperature and mortality in the following sample of dogs:

	Died	Survived	Total
Heated	11	4	15
Cooled	4	6	10
Total	15	10	25

By the method of Example 32, but using Table IB, it can be shown that the lower limit (at $P = 0.025$) for the survival rate in heated animals is 7.8%, while the corresponding upper limit in cooled animals is 87.8%—a difference of 80%. This is a very large possible difference, and the investigator might

say: "Even although the samples show no proof of a real difference, a real difference may nevertheless exist. I shall not be satisfied unless it can be shown that such a possible real difference is unlikely to be more than 10%. How many animals do I require to prove this, if there is in fact no difference?"

Method

To make a precise estimate of the number required is rather complicated, but usually only an approximate estimate is needed. Therefore we can use the fact that, for any fixed probability value, the estimated confidence limits of the difference between two samples varies, approximately, inversely as the square root of sample size. (An inverse square root relationship of similar kind is illustrated in Table VI—see Example 19.)

For the observed samples (total: 25 animals) the difference between the confidence limits is 80%. If there is no real difference, i.e., if the samples belong to the same population, and we increase the sample size, we shall expect still to find no significant difference, and we shall narrow the estimate of the possible real difference. If we multiply the total number of animals by four we shall, approximately, halve the difference (division by $\sqrt{4}$).

Let $N =$ the multiplier required to narrow the difference from 80 to 10%. Then $80/\sqrt{N} = 10$. Therefore $\sqrt{N} = 8$, and $N = 64$. The new total would therefore be $25 \times 64 = 1600$ animals. This is, however, a very rough estimate—see Section C, Note 17.

6. MEASUREMENTS TREATED AS QUALITATIVE STATISTICS

Wherever possible, it is better to make observations by measurements than by qualitative distinctions. Not only does measurement tend to be more objective, but it enables finer distinctions to be made during the observations and when the data are being tested—by comparison of means (two samples), by analysis of variance (two or more samples), or by methods of testing for regression, correlation, or covariance. Sometimes, however, it is desirable or necessary to treat mensuration data as if they were enumeration data, i.e., qualitative statistics.

Example 37

Example 28 presented measurements of visual acuity in a comparison of the effects of prontosil and other treatment; but the data were tested as if they were qualitative statistics. It might be asked why we did not use the actual measurements and compare the mean (average) visual acuity in the prontosil series with the mean visual acuity in the other series. There are two reasons why this is not advisable:

(1). The measurements of visual acuity (6/6 or better, 6/6 to 6/18, 6/18 to 6/60, less than 6/60, eye lost) are not sufficiently precise or uniform.

(2). Even if more precise measurements were available and if the 'eye lost' group were eliminated, we should not know whether a frequency distribution of such measurements would be sufficiently like a normal curve to justify

applying the ordinary tests for mensuration data. Before applying such methods we have to know, either from previous experience of such data or from the sample under observation, that normal curve methods are appropriate.

Example 38

A certain drug, supposed to reduce the incidence of motion sickness, was administered to 104 men who were then tested in a swing. The tests were given to different groups at different intervals after administration of the drug. (We shall assume that the three groups were chosen at random.)

Interval between drug and swing test	No. of men not sick	No. of men sick	Total
One-half hour	41	10	51
One hour	23	7	30
Two hours	12	11	23
Total	76	28	104

Such a table could contain data from many different kinds of investigation; e.g., the headings could be: "Interval between injury and surgical treatment—numbers of surgical successes and failures", "Distance in a tissue from a source of irritation—numbers of migratory cells of Types A and B", "Age groups of cadavera (20-40, 40-60, 60-80 years)—presence and absence of duodenal diverticula". The following remarks would apply to any of them.

It will be seen that in each of the three groups the proportion of men not sick can be expressed as a percentage of the total in that group: one-half hour—80.4%; one hour—76.7%; two hours—52.2%. A graph could now be drawn, with time interval on the x-axis and percentage on the y-axis, and it would show an apparent downward trend of percentages as the interval increased. The common way of testing the reality (significance) of such an apparent trend is by a regression (or correlation) method, but with only three or four sets of values, unless the graph is very nearly a straight line, the trend cannot be proved significant.

The more generally useful method is to test the apparent association between time interval and sickness in the 3×2 contingency table containing the original data (not percentages). Chi square = 6.69. Degrees of freedom = 2. P, from Table VII, is between 0.05 and 0.01. There is therefore a significant association between the incidence of sickness and the time interval. This is perhaps all that need be known in such a problem, but we should note how limited are the inferences from such association tests.

Limitations of Association Tests

All that the association test has shown is that there is a significant difference in the incidence of sickness among the three groups. Two things it cannot show:

(1). The *type* of the association. We should obtain the same chi square value if, without changing the body of the table, we interchanged the headings "one-half hour" and "two hours", although the type of association would be different—longer interval associated with lower incidence of sickness. It is by inspection of the table that we interpret the association as an increasing incidence of sickness with increasing interval; and even then we do not know whether the relation is to be expressed by a straight line or by a curved line as the data seem to suggest.

(2). The *degree* of association. We do not know how closely the sickness incidence and the time interval are related, i.e., whether a short increase of time is associated with a slight increase in the incidence of sickness. From analysis of the 3 × 2 table we do not even know whether there is a significant difference between the one-half-hour group and the one-hour group, or between the one-hour and the two-hours group. (Actually, analysis by fourfold tables reveals no significant differences in either of these pairs.)

The regression method, where applicable, not only establishes an association, but shows its type and degree.

Example 39

Enumeration tests may be useful in preliminary inspection of mensuration data. Fisher (9, Section 24) gives an example of this. Two supposedly soporific drugs, *A* and *B*, were tested on 10 patients, both drugs on each patient. The additional hours of sleep obtained under each drug were recorded, and it was found that in nine patients Drug *B* was apparently more effective; in one patient there was no measurable difference. The most appropriate method of analysis is to find the mean of the 10 differences and test its significance; but the data can also be treated as enumeration data. Of the nine patients who showed an apparent difference between the two drugs, all suggested that *B* was the more effective. If they were equally effective, the true (population) ratio—*A* more effective: *B* more effective—would be 1 : 1. This ratio means a population value of 50% in each class, and Table IA shows that a sample of nine with zero *A*'s has an upper confidence limit of under 50% *A*'s, i.e., among those showing an appreciable difference, *a significant majority obtained a greater increase of sleep after B than after A*.

A test appropriate to mensuration data (Fisher, 9, Section 24) showed that the mean of the 10 differences was significant, i.e., *a significantly greater mean increase in hours of sleep was obtained after Drug B*. The technique of the test is not relevant to this article; but the precise nature of the two conclusions, italicized above, should be carefully noted and contrasted. Observe:

(1). The conclusion from the enumeration test may be all that is necessary in practice.

(2). The mensuration test shows not only that *B* is probably better than *A*, but how much better.

(3). The enumeration test is less sensitive; e.g., as Table IA shows, four patients would be insufficient to prove anything by the enumeration test, but

if the four differences varied very little from each other the mensuration test could prove the mean difference significant.

(4). Because the two tests do not establish precisely the same conclusion, we should not expect the probabilities found by them to be exactly the same.

Example 40

Sometimes measurements, especially in the early stages of an investigation, do not lend themselves readily to analysis by simple methods suitable for mensuration data. The investigator may, indeed, not desire at any stage of his work the full information, e.g., formulae for curves, provided by elaborate methods of analysis, but even in the early stages he will wish to form an estimate of the probable frequency of certain phenomena before deciding to investigate the phenomena further, either by additional observations or by more elaborate statistical methods.

An example of somewhat complex frequency distribution will suffice to illustrate a simple method of treating such problems. Fig. 1 represents bimodal
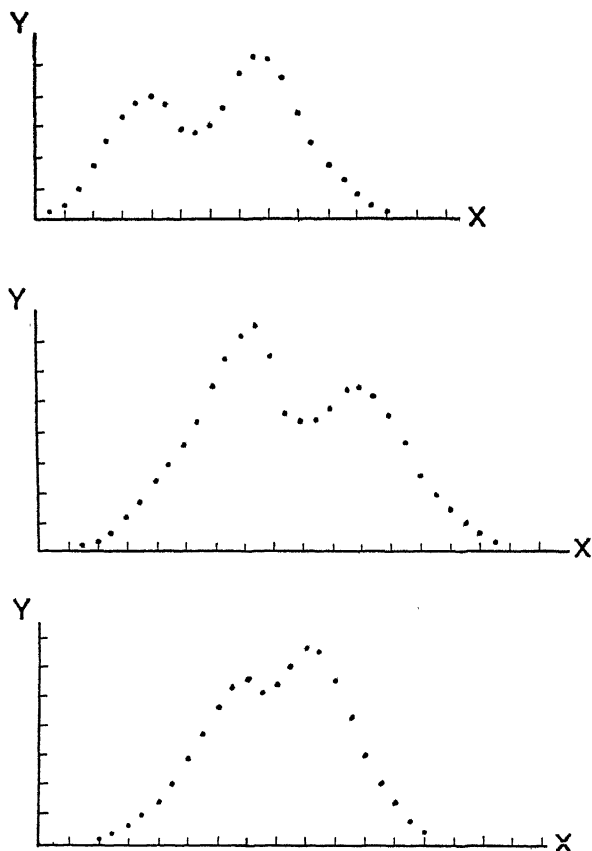
FIG. 1. *Bimodal frequency distributions. The X axis represents attributes, e.g., gastric acidity (mensuration data) or classes of sample (enumeration data). The Y axis represents frequency, e.g., number of persons or number of samples.*

frequency distributions, i.e., with two modes indicated by the two peaks. Such frequency distributions were found at an early stage in an investigation of gastric acidity (27), the measurements of total acidity being the 'measured attributes' in this case. Such bimodal curves suggest that there are really two populations, which, if separated, would form two simple bell-shaped distributions.

A sample from a unimodal population may, however, show bimodality as a result of chance, but the test appropriate to determine this is rather complicated, and, unless the bimodality is pronounced or the sample is large, no evidence of significant bimodality is likely to be obtained. The investigator may continue to accumulate observations that can be pooled to form a large sample, but the type of investigation may not lend itself to this. For example, one may be measuring a small number of blood cells from each of a dozen individuals, or a small number of bacteria from each of 50 different colonies. If each sample represents a different population of individuals the samples should not be pooled; but the frequency of bimodal samples can be easily investigated. From Table IA and IB (Examples 5 and 6) we can conclude that there is a *significant majority* of bimodal samples if all of six samples are bimodal, or eight of nine samples, or 10 of 12 samples, and so on. If, on the other hand, there is no proof that bimodal samples would be likely to occur in more than 5% or 10% of a large population of samples, the investigator may decide that a search for a possible cause is not worth while.

In the investigation of gastric acidity a sample was contributed by each sex–age group among the people surveyed, and a majority of the samples showed bimodality. Further examination of the data revealed that, when the records of persons with no free acid were separated from those with free and combined acid, the frequency distributions for total acidity were all unimodal.

Section C—Notes

NOTE 1—RANDOM SAMPLING VARIATION

The basis of statistical tests is random sampling variation, i.e., the differences between random samples of the same population. It is therefore important to appreciate, from simple examples, how the variation occurs and what variation to expect.

A Random Sampling Demonstration

The demonstration to be described here may seem rather artificial, but it gives a clearer conception of general principles than would an example from the laboratory or the clinic. When an investigator meets a difficult problem of sampling or of interpretation of statistical tests, he will be well advised to visualize it in terms of this sampling demonstration. Indeed, the actual technique of the demonstration is a very useful method of selecting samples in real investigation.

One thousand circular metal-rimmed cardboard disks, 1 in. in diameter, were used. They could be taken to represent human patients, animals, livers from autopsies, red blood cells in a minute (or diluted) drop of blood, or any other collection of individuals, animate or inanimate. The disks were lettered and numbered in various ways, as will be specified later, then placed in a box and mixed thoroughly by taking handfuls and allowing the disks to dribble through the fingers, in the same way as flour and other dry ingredients are mixed in breadmaking. After 8 or 10 such movements the disks were poured into another box and mixed again, and then, to obtain a sample of, say, 20 individuals, a handful was taken and 20 disks were counted without inspection of their letters or numbers. The remainder of the handful was then returned to the box, as was the sample when its composition (letters and numbers) had been recorded. The thousand disks were then thoroughly mixed again before the next sample was taken.

Samples of Two Individuals

If there are equal numbers of disks marked M and F (males and females) in the population and if, taking a number of random samples, two disks per sample, we look at the disks one after the other, we expect, from our knowledge of chance, that the first disk looked at will be M in about half the samples and F in about half the samples. We expect also that, of all the samples in which the first disk is M, about half will have their second disk M also, while half will have their second disk F. Similarly, among the samples with first disk F, there will be approximately equal numbers with second disk M and second disk F. In diagram form:

Second disk M (one-quarter of the samples)

First disk (half the samples) M

Second disk F (one-quarter of the samples)

Second disk M (one-quarter of the samples)

First disk (half the samples) F

Second disk F (one-quarter of the samples)
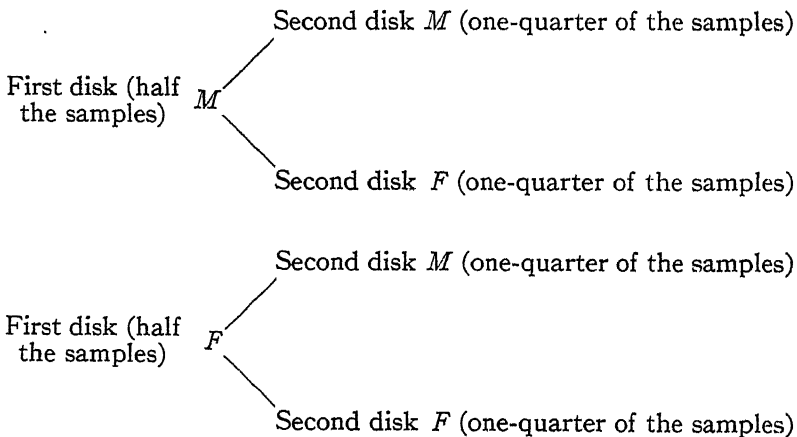
The order (first or second) in which the disks are examined is, of course, immaterial, and the results can be expressed thus:

Type of sample	MM	MF	FF
Relative frequency	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{1}{4}$
	0.25	0.50	0.25

We know, again from our experience of chance, that, if we take only a few samples, they may differ considerably from these proportions, but if we took more and more samples and they continued to differ we should become more and more suspicious, either that the original ratio of M to F was not 1 : 1, or that there was bias in the sampling. If there is no bias we expect the proportions to approach closer and closer to those deduced from our knowledge of the population. Some actual experiments showed the following:

Type of sample (two disks each)	Relative frequencies			
	First 10 samples	First 100 samples	First 500 samples	1000 samples
MM	0.30	0.26	0.21	0.23
MF	0.40	0.44	0.51	0.51
FF	0.30	0.30	0.28	0.26

A Population of Samples

Such a process of sampling gives us a population of samples, and we can imagine the process continuing indefinitely, to produce an *infinite population*, i.e., a population that can be made as large as one likes. As the process continued the relative frequencies would approach closer to the values 0.25, 0.50, 0.25; and these can be called the '*true*' *values*, or the values for the infinite population. For brevity one can speak of *true (population) values*, defined as values that would be approached closer and closer by continued random sampling. (The approach is, of course, not steady as in many mathematical series that approach a limit. It fluctuates as samples vary, but its main line continues.)

Recalling the terms "probability", "odds", and "chances" from Section A, we see that the relative frequencies (0.25, 0.50, and 0.25) are obviously *probabilities*. They can be expressed also as *percentage frequencies: 25%, 50%, 25%*. Thus, the probability of obtaining a random sample of Type *MM* is 0.25, i.e., we should expect 25% of random samples to be of that type. The *odds* against finding an *MM* sample are 3 to 1, i.e., there is one chance in four of finding such a sample.

Note 2—The Binomial Expansion

Turning again to the population of 1000 disks (Note 1), we could use the same process of reasoning, based on our experience of chance, to work out the relative frequencies of M and F disks in any size of sample, and we could do likewise in experiments where there were 700 disks marked R (recoveries) and 300 disks marked D (deaths), i.e., for probabilities of 0.7 and 0.3; and similarly for any other classes of marked disks, e.g., for probabilities of 0.997 and 0.003. For samples containing more than three or four individuals, however, and for unequal probabilities, the step-by-step calculation from first

principles is lengthy and laborious. Therefore we use a formula, *the binomial expansion*. This is not a mere approximation to the step-by-step method and it does not introduce any additional assumptions. Using the same kind of reasoning as in the step-by-step method, the mathematician can show that the binomial expansion gives the required results for any size of sample and for any pair of probabilities in a twofold classification such as we are discussing (note the derivation of 'binomial'—*bi-*, double; *nomen*, name).

A Simple Binomial Expansion

Medical investigators seldom need to use the binomial expansion directly, because tables derived from it (or approximating to it) are available; but they should know how it works in a simple case, e.g., the M and F disks in our demonstration.

The formula is $(p + q)^N$ where—

p = the probability of meeting an M disk;

q = the probability of meeting a not-M disk, i.e., an F disk;

N = the number in the sample, here 2.

$$(p + q)^N = (p + q)^2 = p^2 + 2pq + q^2 .$$

The superscript numerals indicate the composition of the samples, e.g., p^2 refers to samples with two individuals, each of whose probability is p, i.e.,

TABLE I

Class of sample—20 disks in each		Percentage of R in sample	Probabilities from the binomial $(0.7+0.3)^{20}$		Percentage frequencies found experimentally in different series of samples			
R	D		Actual	Converted into %	1st 10	1st 100	1st 500	1000
0	20	0	0.000	0.0	—	—	—	—
. .	.	.	. . .	. .	—	—	—	—
.	.	.	. . .	. .	—	—	—	—
6	14	30	0.000	0.0	—	—	—	—
7	13	35	0.001	0.1	—	—	—	—
8	12	40	0.004	0.4	—	—	—	0.2
9	11	45	0.012	1.2	—	3	1.6	1.5
10	·10	50	0.031	3.1	10	4	2.8	3.1
11	9	55	0.066	6.6	—	7	8.2	6.8
12	8	60	0.114	11.4	10	13	14.0	12.3
13	7	65	0.164	16.4	40	19	15.8	16.6
14	6	70	0.192	19.2	10	23	20.8	20.6
15	5	75	0.179	17.9	10	12	13.6	15.7
16	4	80	0.130	13.0	20	13	13.2	12.9
17	3	85	0.072	7.2	—	5	6.6	6.8
18	2	90	0.028	2.8	—	1	2.4	2.8
19	1	95	0.007	0.7	—	—	1.0	0.7
20	0	100	0.001	0.1	—	—	—	—
Total			1.001	100.1	100	100	100.0	100.0

Note.—0.000 indicates a probability less than 0.0005. By using sufficient decimal figures we should find a value for each class, progressively diminishing toward the tail of the distribution.

two M disks.   The quantity pq, i.e., p^1q^1, refers to samples with one M disk and one F disk; q^2 refers to samples with two F disks.

In the present instance $p = \frac{1}{2}$, $q = \frac{1}{2}$.   Substituting these values in the formula, we see how the appropriate probabilities are reached:

$$p^2 + 2pq + q^2 = (\tfrac{1}{2})^2 + (2 \times \tfrac{1}{2} \times \tfrac{1}{2}) + (\tfrac{1}{2})^2 = \frac{1/4}{MM} + \frac{1/2}{MF} + \frac{1/4}{FF}.$$

A More Difficult Binomial Expansion

There were 700 R disks (70%; probability $= 0.7$) and 300 D disks (30%; probability $= 0.3$).   If we take random samples of 20 disks each, what are the probabilities of the various classes of sample?

$$p = 0.7; \; q = 0.3; \; N = 20. \quad (p + q)^N = [\,(0.7) + (0.3)\,]^{20}.$$

The results of this expansion give us a population of samples (20 disks per sample) and are set out in Table I.   For comparison the results of some actual disk sampling experiments are shown.   Fig. 2 is a graph of the true probabilities converted to percentage frequencies.
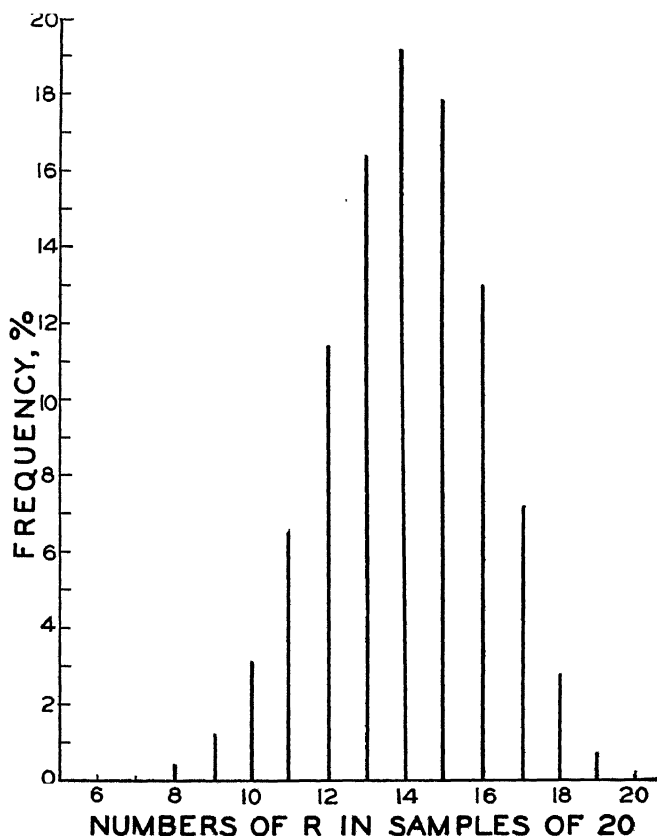
FIG. 2. *Graph of percentage frequencies from Table I, obtained by expansion of* (0.7 + 0.3)²⁰.

A table or graph that shows how many items, e.g., individuals or samples, occur in the various classes is called a table or graph of *frequency distribution*. In frequency distributions the value for the class that contains most items is called *the mode*—the 'most fashionable' value (French, *la mode*, the fashion). It is indicated by the peak of frequency graphs.   As would be expected, the mode in the present example is 70% *R*, the true percentage for the original population of 1000 disks.   On each side of the mode the graph slopes down to form a *tail*, extending to the ends of the distribution.

NOTE 3—ARGUMENT FROM SAMPLE TO POPULATION

In medical research we meet, as a rule, not populations but samples, and, in one form or another, the questions arise: To what populations may this sample belong, and to what populations is it unlikely to belong?   To answer those questions we can, as in Note 2, work out the random sampling distribution for any population, and then, if our sample would be found only rarely in a certain population, we can say: "It is unlikely to belong to that population."   If the sample would be found often in certain other populations we can say: "It may well belong to one of those populations; we have not sufficient reason for believing that it does not."

Let us suppose that we did not know about the markings on the thousand disks, except that each was marked either *R* or *D*, and let us suppose that we obtained by random selection a sample of 20 with 9 marked *R* and 11 marked *D*.   In Table I and Fig. 2 some classes of sample are relatively rare, i.e., of low probability.   In the left-hand tail, samples of the class 9 *R* and 11 *D*, and the still rarer classes (8 *R*, 12 *D*,   7 *R*, 13 *D*; etc.) have a total probability of $0.012 + 0.004 + 0.001 = 0.017$; i.e., less than 0.025.   In other words, this group of samples forms less than $2\frac{1}{2}\%$ of the total samples.   The capital letter '*P*' is commonly used to designate such probabilities, and, in cases like the present one, *P, for any particular type of sample in a population, is the combined probability of finding, by random sampling, such samples and also all samples that are farther out in the same tail of the distribution.*

The question, it will be seen, is not: "What would be the probability of the particular type of sample?"   Instead, we ask: "Would the sample belong to a rare group (a group with low probability), i.e., would the sample be far out in a tail?"   Since our sample of 9 *R* and 11 *D* (45% *R*) would be in a group that would rarely be found by random sampling from a population of 70% *R*, it would obviously be safer to believe that the sample belongs to a population with a lower proportion of *R* than 70%; i.e., we feel justified in believing that, if we took more and more samples, we should approach some value lower than 70%.   'Rarity' in this connection is usually taken to mean a *P* value of less than 0.025—see Note 4.

It may at first be somewhat difficult to understand why we consider not only the probability of the observed sample but also the probabilities of rarer samples.   A full explanation is undesirable here, but one reason will be obvious if we visualize a distribution that slopes more slowly than in the present series,

i.e., has long tails with many types of sample, each type with a rather low probability. If we rejected each of these types because of its own probability we should, altogether, reject a considerable proportion of the total population.

NOTE 4—SIGNIFICANCE AND NONSIGNIFICANCE

As stated in Section A3, in a problem like that of Note 3, $P = 0.025$ is by convention taken as the boundary between significant and nonsignificant differences, and $P = 0.005$ is taken as the boundary between significant and highly significant differences. What this implies is seen from Table I (p. 68) and Figs. 2 and 3. In Note 3, assessing a sample with $9R$ out of 20 (45%)
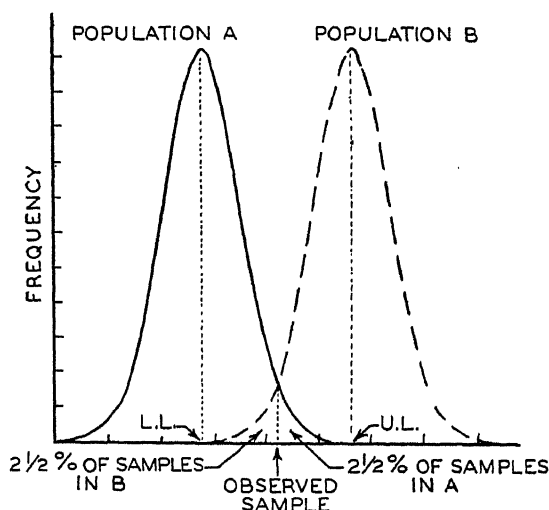
FIG. 3. *To illustrate the conception of confidence limits. L.L. = lower limit. U.L. = upper limit.*

we cut off the tip of the left-hand tail containing not more than $2\frac{1}{2}\%$ of the samples. Likewise, if we were assessing a sample containing more than 70% R, we should cut off the right-hand tail containing, at the most, $2\frac{1}{2}\%$. Applying these standards throughout our work, we cut off 5% of samples (1 in 20), i.e., our level of significance, from both tails, is 5%, or P from both tails = 0.05, or the odds against finding by random sampling one of our 'rare' samples are 19 to 1.

When we set $P = 0.005$ as the boundary between significant differences and highly significant (or very significant) differences, we cut off $\frac{1}{2}\%$ of the samples in each tail, i.e., a total of 1% of samples. Our level of significance, from both tails, is therefore 1%, or P from both tails = 0.01, or the odds against finding by random sampling one of our 'very rare' samples are 99 to 1.

If the reader will consider these statements carefully he will avoid being confused by the apparent conflict between $P = 0.025$ and $P = 0.05$ as standards of significance and similarly by the apparent conflict between $P = 0.005$ and $P = 0.01$.

NOTE 5—REASONABLENESS OF SIGNIFICANCE CONVENTIONS

The 5% and 1% Criteria of Significance

These two standards of significance are, of course, arbitrary and conventional, and anyone is at liberty to set his own standards, but the conventions seem reasonable when their implications are noted. They apply both when we are testing a sample against a known or hypothetical population value, and when we are comparing samples with each other.

First let us consider all of our investigations in which there is no real difference, i.e., where chance alone is responsible for the difference between our sample and the particular population value that we are postulating, or between samples that we are comparing with each other. If we accept the 5% level of significance throughout we shall in 95% of these investigations correctly proclaim no significant difference. If these judgments are considered as 'diagnoses', the standard of accuracy will probably be admitted as a reasonable one.

In our other investigations, in which a real difference does exist, there will be many in which we shall, accepting the 5% level, diagnose correctly, but there will, of course, be some cases where, although a real difference exists, we shall proclaim a nonsignificant difference. If, however, we tried to reduce this error by accepting $P = 0.1$ as the criterion, we should run the risk of a 10% error in all those investigations where there was no real difference. If, on the other hand, we waited until P was 0.01 or 0.001 before accepting a difference as significant at all, we should run the opposite risk of rejecting real differences in many investigations.

As some of the examples in Section B indicate, an observer may feel justified in continuing an investigation when the samples already observed indicate odds of less than 19 to 1. In other cases, he may demand odds of 100 to 1 or higher, e.g., when he wishes to feel confident that a certain drug will produce unfavorable reactions in not more than a certain small proportion of people. Note.—The only safe way to avoid being swayed by the results of an investigation, is to set one's standard of significance at the outset.

One-sided Comparisons

Some special consideration should be given to judgments that can be called "one-sided comparisons", in which the observer is concerned with differences in one direction only, i.e., with only one tail of a distribution.

For example, he might test a treatment for a certain disease by taking 20 pairs of animals (litter mates), applying the treatment to one member of each pair and leaving the other member as an untreated control. If he knew that the treatment could not impede recovery but found that in more than 10 of his pairs the control animals recovered better than the treated animals, he would attribute the difference to random sampling variation, and would not apply a test of significance. He would, in fact, never run the risk of erroneously proclaiming as significant any differences in this direction; and he would therefore be justified in setting $P = 0.05$ and $P = 0.01$ as his criteria of

significance in the other direction, i.e., for samples in which treatment appeared better than no treatment, for by so doing he would insure that his erroneous judgments would not exceed 5% (or 1%) of his total judgments in this type of investigation.

Similarly, when comparing mortality in samples of treated and untreated animals, if the investigator knew that the treatment could do no harm, he would attribute to random sampling variation all cases in which treated animals had a higher mortality than untreated animals. This again is a 'one-sided' comparison, in contrast to the 'two-sided' comparison in which the observer would wish to know whether treatment or no treatment was better, or which of two treatments was better (see Section A4). In the one-sided comparison, since he would make erroneous judgments of significance in the one direction only, he might decide to set as his standards $P = 0.05$ and $P = 0.01$, where P is the probability of finding samples that showed a mortality difference *in favor of the treatment* as great as in the observed samples, and greater.

To have presented in our tables information on probabilities for one-sided as well as two-sided comparisons would have entailed doubling most of the· tables; and therefore, in most instances, the two types of comparison are treated in our examples as if they were the same. The tables and other calculations give us tests of significance for two-sided comparisons and we apply the same verdict even when the comparison is in fact one-sided. We are therefore taking $P = 0.025$ and $P = 0.005$ from a single tail as the standard of significance in both cases, and we are thereby merely setting our standards somewhat higher for judgments in which differences in only one direction are of interest—a potential error of $2\frac{1}{2}\%$ (or $\frac{1}{2}\%$) instead of 5% (or 1%). For instances of one-sided comparisons see especially Examples 6 and 14.

NOTE 6—CONFIDENCE LIMITS

The technique and rationale of finding confidence limits are given briefly in Section A3, and the process is represented pictorially in Fig. 3 (p. 71) by two frequency distributions, symmetrical for convenience in drawing. They are similar to Fig. 2, but the tips of the ordinates (vertical lines) have been joined and most of the ordinates themselves have been omitted.

For the *lower* limit, the problem is to find a population A, such that our observed sample falls at the dividing line between the *upper* $2\frac{1}{2}\%$ of the samples and the rest of the samples. The true (population) percentage is indicated by the mode or peak of the frequency distribution (at 70% R in Fig. 2). This percentage is the required lower confidence limit at the $2\frac{1}{2}\%$ level, i.e., for $P = 0.025$.

Similarly, for the *upper* limit we have to find a population B, such that our observed sample falls at the dividing line between the *lower* $2\frac{1}{2}\%$ and the rest of the samples. The mode of this distribution will ·be the required upper

confidence limit at the $2\frac{1}{2}\%$ level, i.e, for $P = 0.025$. The space between the upper and lower limits is a *confidence belt.*

For confidence limits at the $\frac{1}{2}\%$ level, i.e., for $P = 0.005$, we should push the two populations, A and B, farther apart, i.e., enlarge the confidence belt.

In any actual problem, confidence limits could be found directly from binomial expansions, by trying various values of p and q in the expression $(p + q)^N$, where N is the total number of individuals in the sample; but this is very laborious.

A commonly employed method, although much simpler, is often misleading unless proper corrections are made. It is based on the normal curve (Note 7) and uses the standard deviation (Note 8). To provide more accurate estimates, Tables IA, IB, and II, and Graphs 1 to 6 ($=$ Figs. 6 to 11) were prepared.

NOTE 7—THE NORMAL FREQUENCY CURVE

The normal curve is a widely used approximation to the binomial expansion, and when carefully employed it is very valuable. In this connection the term 'normal' is used, not in contrast to 'abnormal', but in the sense of a 'standard' or measuring device (Latin, *norma*, a carpenter's square).

The development of the normal curve from the binomial expansion $(p + q)^N$ is pictorially presented in Fig. 4—frequency distributions with increasing size of sample (N). Where $p = q = \frac{1}{2}$, as in Note 2, the binomial expansion is symmetrical from the first, as is the normal curve, but even when p and q are unequal the distributions approach closer and closer to the normal curve when N is increased.

When a mathematician establishes the formula for the normal curve he is, in essence, proving the fact that is suggested by Fig. 4. The formula itself is seldom used in the everyday application of statistical methods because probability tables, derived from the formula, are available.

NOTE 8—THE STANDARD DEVIATION, $\sqrt{Npq}$

In any distribution, either of measurements or enumeration data, the variation, i.e., the amount of spread of the distribution, is expressed by a quantity called the *standard deviation*, the word 'deviation' being equivalent to 'variation'. (Sometimes the term *standard error* is used instead of 'standard deviation', the term 'error' being again equivalent to 'variation'.)

For distributions of measurements the standard deviation has to be estimated from the measurements themselves, but for a binomial distribution, as in Table I (Note 2), it can be found from the formula $\sqrt{Npq}$, where $N =$ total number of individuals in the sample, $p =$ probability of occurrence of one type of individual, A; $q =$ probability of occurrence of the other type, not-A.

In Table I, $N = 20$; $p =$ the probability of recoveries (R) $= 0.7$; $q =$ the probability of deaths (D) $= 0.3$. Standard deviation (S.d.)

$= \sqrt{20 \times 0.7 \times 0.3} = \sqrt{4.20} = 2.05$ out of 20, i.e., 10.25%. The validity of the formula $\sqrt{Npq}$ can be proved mathematically, but the proof is unnecessary for investigators. Its accuracy can be illustrated by treating any

$$N=7$$
$$(0.5+0.5)^7$$

$$N=50$$
$$(0.5+0.5)^{50}$$

$$N=100$$
$$(0.9+0.1)^{100}$$
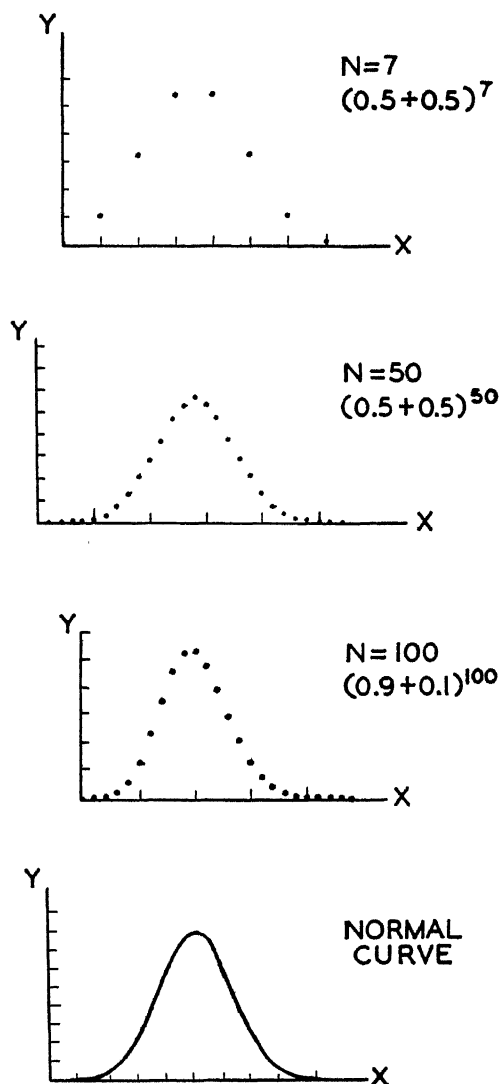
NORMAL CURVE

FIG. 4. *Genesis of normal curve from binomial expansion with increasing size of sample (N). The X axis represents class of sample as in Fig. 2. The Y axis represents probabilities or percentage frequencies.*

binomial expansion such as that of Table I (Note 2) as a series of measurements and estimating the standard deviation by direct calculation.

If percentages are used throughout, the formula $\sqrt{Npq}$ becomes $\sqrt{A\,(100-A)/N}$, where A is the percentage of Class A in the population.

For Table I this becomes $\sqrt{70 \times 30/20} = 10.25\%$. Therefore twice the standard deviation = 20.50%.

Now in a normal curve, if we measure from the center (mean) of the distribution, twice the standard deviation, we exclude in each tail rather less than $2\frac{1}{2}\%$ of the total distribution. (1.96 S.d. cuts off almost exactly $2\frac{1}{2}\%$.) Applying this to the binomial distribution in Table I (Note 2) we measure off from 70% 2 S.d., i.e., 20.50, to give: $70 + 20.50 = 90.50\%$ R; $70 - 20.50 = 49.50\%$ R. A sample with 45% R (i.e., 9 R and 11 D) is therefore beyond twice the standard deviation away from 70%, and if we met such a sample and knew nothing about its population, we could, by means of the standard deviation of a 70% population, decide that the sample percentage was significantly different from 70%. Two and one-half times the standard deviation (more exactly 2.57 S.d.), similarly used, excludes approximately $\frac{1}{2}\%$ of the distribution in each tail; therefore it can be employed to determine highly significant differences.

Used in this way, to test a sample against one particular population value the standard deviation is a simple and often sufficiently accurate method. To use it for the estimation of confidence limits requires trial of several different population values, and the final result contains an unknown degree of error. The standard deviation method, moreover, becomes very undependable: (1) as samples become smaller; (2) as distributions become more skew (asymmetrical), i.e., as p and q become more unequal; (3) when, as is often done, the standard deviation is calculated from the sample itself. Three examples may be given:

Example (1)

In a sample of 100 are eight color-blind men. Does this differ significantly from a population percentage of 4?

(a). Standard deviation estimated from population value $= \sqrt{4 \times 96/100} = \sqrt{384}/10 = 1.96\%$. Sample percentage *minus* population percentage = 4. 4/S.d. = 4/1.96 = 2.04, which is large enough to indicate a significant difference.

(b). Standard deviation estimated from the sample value $= \sqrt{8 \times 92/100} = \sqrt{736}/10 = 2.713$. Sample percentage *minus* population percentage = 4. 4/2.713 = 1.5, showing no evidence of significance. The lower confidence limit ($P = 0.025$) estimated in this way would be 8 − 2 S.d. = 8 − 5.426 = 2.574%.

(c). Table IB (number of A's = 8; N = 100) shows that the lower confidence limit ($P = 0.025$) is 3.5%. There is no significant difference between the sample value and a population percentage of 4, but the proximity to significance is much greater than the standard deviation, estimated from the sample, would suggest, for the lower limit ($P = 0.025$) so estimated (2.574%) is lower than Table IB shows even for $P = 0.005$, viz., 2.6%.

Example (2)

In Example 26 of Section B, among 56 mental defectives, 14 (25%) had mothers with *Rh* negative blood. Is this significantly different from a population percentage of 15?

S.d. $= \sqrt{15 \times 85/56} = \sqrt{1275/56} = \sqrt{22.77} = 4.77$.

Sample percentage *minus* population percentage, divided by S.d. $= (25 - 15)/4.77 = 2.10$ — significant.

Tables derived from the normal curve show that the probability of finding samples in one tail beyond 2.10 S.d. is 0.0179, indicating odds of 9821 to 179, or about 55 to 1. By contrast, Table IB (number of *A*'s $= 14$; *N* between 55 and 57) shows the lower limit ($P = 0.025$) to be between 14.7 and 14.1%. The sample has not quite reached the level of significance, and this judgment is confirmed by calculating the exact *P* from the binomial expansion, i.e., 0.0343, almost double the value derived from the standard deviation, and corresponding to odds of only about 28 to 1.

This example shows that, for a given criterion of significance (here $P = 0.025$ for one tail), the use of the standard deviation may give a verdict of significance, whereas the true verdict is one of nonsignificance.

Note in this instance the value of finding the exact *P*. The investigator may justifiably claim that there is no reason to suppose that mental defectives might tend to have a *lower* proportion of mothers with *Rh* negative blood than occurs in the general population, i.e., he is interested in a 'one-sided' comparison (see Note 5) and would be satisfied with $P = 0.05$ from the one tail of the distribution—odds of 19 to 1 against finding by random sampling of the general population (15% *Rh* negative), samples containing 25% and more *Rh* negative persons. On that basis he would accept the result ($P = 0.0343$) as indicating a significantly higher percentage.

Example (3)

In a sample of 20 there are three *A*'s (15%). The standard deviation, estimated from the sample, is $\sqrt{15 \times 85/20} = 7.98$. The lower confidence limit ($P = 0.025$), estimated from the standard deviation $= 15 - 2 \times 7.98 = $ *minus* 0.96, an obviously impossible value. The lower limit at $P = 0.005$, by use of $2\frac{1}{2}$ S.d., would appear to be $15 - 19.95 = $ *minus* 4.95%. Table IB shows that the limits are respectively 3.2 and 1.8%.

These three examples have shown that the use of the standard deviation of the binomial distribution, without a correction term, can be very misleading; and there is no simple rule whereby the investigator can tell when not to trust the method. Hence the value of tables such as IA, IB, and II.

NOTE 9—CHI SQUARE USED FOR TESTING A SAMPLE AGAINST A POPULATION VALUE

In Example 13 there were 50 individuals with reactions after inoculation: mild, 13; moderate, 17; severe, 12; very severe, 8. The hypothesis to be tested was that the true (population) ratio was $1:1:1:1$, indicating an

equal probability, $\frac{1}{4}$ or 0.25 in each class.   To test this we need to find whether the observed sample would occur rarely or commonly among random samples of 50 from a population with the 1 : 1 : 1 : 1 ratio.   The relative proportions of samples could be exactly ascertained by the expansion of $(\frac{1}{4} + \frac{1}{4} + \frac{1}{4} + \frac{1}{4})^{50}$, which is like the binomial but is called a 'multinomial' expansion because it contains more than two classes.   This would be a long process, and we can usually obtain a sufficiently accurate result by means of chi square, as in Example 13.

By following the rules of procedure one can safely apply the chi square test without probing more deeply into its nature; but those who wish to form some idea of what is being done in the test can perhaps be helped in the following way.

First, let us imagine that we had a large population in which the numbers of individuals in the four classes, labeled as in our sample, were equal (a 1 : 1 : 1 : 1 ratio), that we took a large number of samples of 50 and arranged a graph of the frequency distribution.   It would be more complicated than the distribution in Fig. 2 (Note 2) because there would be four classes instead of two; but for simplicity let us imagine it represented by such a graph.   The samples that had ratios nearest to 1 : 1 : 1 : 1 would be most numerous and would form the hump of the graph; those that were most unlike that ratio would be much rarer and would lie in the tails of the graph.   Now let us suppose that chi square values, with the ratio 1 : 1 : 1 : 1 as the hypothetical ratio, were estimated for each of the samples.   The greater the discrepancy from this ratio, the larger would be chi square and the rarer would be its occurrence, i.e., the high chi square values would be in the tails of the distribution.

Next, we can suppose that the actual frequency distribution was replaced by a normal curve (Note 7).   From the equation for the normal curve we could indicate the frequency with which the various values of chi square occurred.   In fact, although the process is not nearly so simple as this, it is from the normal curve that probability tables of chi square are derived.

Note that $P = 0.05$ and 0.01 are used in assessing significance by chi square, not, as in Tables IA, IB, and II, $P = 0.025$ and 0.005.   This will be best understood from a simpler example in Note 11, and then applied to the more complicated problem discussed here.

Finally, it should be observed that there is no need for separate tables of chi square related to size of sample or to different ratios (1 : 1 : 1 : 1; 9 : 3 : 3 : 1; etc.), because these characteristics have already entered into, and have been allowed for by, the calculation of chi square itself.

NOTE 10—LIMITATIONS OF THE CHI SQUARE TEST

As stated in Note 9, probability tables for chi square are based on the normal curve, which is a continuous and symmetrical distribution.   Therefore there are two factors that render probabilities derived from chi square inexact

estimates of the true probabilities obtained from binomial or multinomial expansions: (1) Continuity, (2) Symmetry.

Continuity.—If chi square is used for samples that contain only two classes, Yates's simple correction for continuity can be applied (Notes 11 and 14)—see Fisher (9), Section 21.01. Where there are more than two classes, and when contingency tables are larger than fourfold, the correction is not applicable, but the error is less serious then.

Symmetry.—As has been exemplified for binomial distributions, the graphs of exact probabilities are, except with 1 : 1 ratios, asymmetrical. Increase of sample size reduces the error of chi square from this factor, but the important feature is not total size of sample but the size of the theoretical (t) values.

These limitations dictate the precautions specified in Examples 13, 20, and 27 of Section B.

NOTE 11—CHI SQUARE USED WITH TWO-CLASS SAMPLES

Even when there are only two classes in a sample, chi square is often used in testing the sample against a population ratio, e.g., 1 : 1, or 3 : 1, or 5 : 2. For this purpose of course, Tables IA, IB, II, and III are not only simpler but more accurate than chi square. However, with samples large enough for both t values to be 10 or more, the chi square probability does not depart so widely from the exact (binomial) probability as to be misleading in tests of significance, and it is perhaps desirable to exemplify this use of chi square, especially because it involves the 'correction for continuity', which is met also in the application of chi square to contingency tables.

Correction for Continuity

In the calculation of chi square for two-class samples the only difference from the calculation for samples with more than two classes (see Example 13) is that each of the two ($a - t$) values is reduced by half a unit (0.5). This is called the 'correction for continuity', and the resulting chi square is called 'chi square corrected for continuity' (χ_c^2). The correction makes the probability derived from chi square, i.e., based on the normal (continuous) curve, more like the exact probability that would be derived from the binomial distribution, a discontinuous series. Note.—The correction is a reduction in size, regardless of the sign, positive or negative, of ($a - t$).

Method of Applying the Chi Square Test

A sample of 50 contains 32 X's and 18 Y's. Is it unlikely that the population value was 50%, a 1 : 1 ratio? We proceed as follows:

	X	Y
a	32	18
t	25	25
$(a \sim t)$	7	7
$(a \sim t) - 0.5$	6.5	6.5
$(a \sim t - 0.5)^2$	42.25	42.25
$(a \sim t - 0.5)^2/t$	1.69	1.69

(The symbol $\sim$ indicates 'difference between', regardless of sign, $+$ or $-$.)

Chi square (corrected for continuity) $= 1.69 + 1.69 = 3.38$.

Note.—For a $1:1$ population ratio, as here, $\chi_c^2 = 2 (a \sim t - 0.5)^2/t$.

Degrees of freedom $= 2 - 1 = 1$. Table VII shows that the lowest significant value $(P = 0.05)$ for one degree of freedom is 3.841. Therefore our sample shows no significant difference from a population ratio of $1:1$. Evaluated more precisely from the Appendix Table 4B of Yule and Kendall (30), or by interpolation in our Table VII (see Fisher (9), Section 21.1 for method), $P = 0.0660$.

P and $\frac{1}{2}$ P from Chi Square

In assessing significance by chi square we commonly use $P = 0.05$ and 0.01 as standards, not $P = 0.025$ and 0.005. To elucidate this, let us test the present sample by the binomial expansion $(p + q)^{50}$, where $p = q = \frac{1}{2}$. The tail containing the observed sample runs:

X	Y	Probability
32	18	0.0160
33	17	0.0087
34	16	0.0044
35	15	0.0020
36	14	0.0008
37	13	0.0003
38	12	0.0001
39	11	0.0000
.	.	.
.	.	.
.	.	.
50	0	0.0000

$$P = 0.0323$$

The opposite tail of the same distribution contains samples as rare as those in the tail where the sample lies, and, because the distribution is symmetrical, the values are the same: $X = 18$, $Y = 32$—0.0160; $X = 17$, $Y = 33$—0.0087; etc., giving a total of 0.0323. Now chi square represents both tails; i.e., P from chi square is the probability of finding *all* samples that are as rare as, and rarer than, the observed sample. It takes into account the samples that differ from the hypothesis in the same direction as the observed sample, and also those that differ in the opposite direction. To get the probability of the one tail in which the sample lies, we can take $\frac{1}{2}P$. Thus, $P = 0.0660$, $\frac{1}{2}P = 0.0330$, which is not far from the exact value, 0.0323.

Applied to the more complicated problem in Note 9, we can say that chi square includes (1) the samples, like the observed sample, with excess of 'mild' and 'moderate' reactions, and (2) samples that have excess of 'severe' reactions.

NOTE 12—THE GENERAL PROCEDURE IN CONTINGENCY TESTS

The general procedure in contingency tests can be illustrated from the data of Example 15:

	R	D	Total
Treatment *V*	2	7	9
Treatment *W*	5	1	6
Total	7	8	15

Whatever particular method is used—the exact (factorial) method (Note 13) or chi square (Note 14)—the procedure can be outlined as follows:

(1). Assume that the two samples are random samples from the same population.*

(2). Find from the available information, i.e., the two samples, the best estimate of the composition of the assumed population. From the above table the best estimate of the population ratio *R : D* would be 7 : 8. (This procedure is comparable to the ordinary practice of getting the best estimate from two or more measurements by adding them together and finding their average.)

(3). Calculate how often pairs of samples of the same size as the observed samples and showing the same and greater differences would be met in random sampling of the one population. More precisely, calculate *P*, the probability of finding, by random sampling, pairs of samples that are as rare as, and rarer than, the observed samples.

(4). If *P* is low, indicating that such pairs of samples would rarely be obtained from the same population, accept it as a proof that the populations are different, and call the difference between the samples 'significant', or if *P* is very low call the difference 'highly significant'. If *P* is not low, call the difference 'not significant', indicating that there is not sufficient evidence to show that the samples have come from different populations. (For conventional standards of significance, see Section A4 and Notes 13 and 14.)

Alternatively, of course, the verdicts can be expressed in terms of 'homogeneity' and 'association' (Section A4).

Dangers in Interpretation of Contingency Tests

It is important to note the wording of the verdicts just given. For example, a nonsignificant difference is not a proof that the samples have come from the same population. Particular care should be taken with the word 'association', which does not imply causation. We may have concluded that a significant association is present, but we have not thereby proved, or even suggested, a

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* *Strictly, we are dealing with the same population of samples in the exact (factorial) test, and with the same population of individuals in the chi square test; but this distinction is not important for an elementary grasp of the general procedure.*

cause-and-effect relation.   If there is an association between X and Y, X may be the cause of Y or vice versa, or both may be due to some common cause, or the relation may be more indirect and complex.

When time is a factor, associations can be very misleading.   For example, a survey of the past forty years would doubtless show an increase in (a) consumption of cigarettes, (b) reported mortality from coronary artery disease, and (c) travel by air; and an association between these three could therefore doubtless be established.   We should not think of attributing (c) to (a), or (a) to (c), and the mere proof of association between (a) and (b) gives no more reason for suspecting a causal relation between those two phenomena, however plausible such a relation may seem.

Contingency tests, like all other tests of significance, show only how far chance could account for the results.   The interpretation is left to the observer.

NOTE 13—THE EXACT METHOD FOR ·CONTINGENCY TESTS

The exact method (Fisher (9), Section 21.02) looks complicated and laborious, but can be easily carried out by anyone who can use logarithms. It is the method that (1) provides exact probabilities, to which the probabilities derived from chi square are an approximation; (2) tests the accuracy of the chi square method; and (3) was used for the construction of Tables IV, V, and VI.

The exact method starts with a display of fourfold tables showing all the possible pairs of samples that could be found under the imposed conditions (see Note 12), namely, that the sizes of samples be the same as in the observed samples, and that the composition of the population (the population ratio), estimated from the observed samples, remain constant.   These conditions are realized by keeping the subtotals in all the tables the same as in the original table.

The random sampling probabilities of the various possible pairs of samples are then found from a formula; but it should be noted that this formula is based on experience, just as is the use of the simple binomial expansion $(p + q)^2$ discussed in Note 2.   Like the binomial expansion, the formula involves the use of factorials (see Note 22 and use Table VIII).

The data of Note 12 are:

	R	D	Total
Treatment V	2	7	9
Treatment W	5	1	6
Total	7	8	15

The compartments in the body of the table, occupied by 2, 7, 5, and 1, are *cells*.   The subtotals, 7, 8, 6, and 9 are *marginal totals*; 15 is the *grand total*.

The main procedure will be summarized later, but the detail of the following seven stages should be noted first:

(1). With the marginal totals and grand total constant, display all the possible arrangements of numbers in the cells. The simplest way is to begin by making all the possible changes in the row or column that contains the smallest marginal total (here the second row), running up and down from the observed samples. Then change the other cells correspondingly. For reference, we letter the sets of pairs.

Possible pairs of samples   Possible pairs of samples

(a)   1 | 8   (e)   5 | 4
       ——|——   ——|——
       6 | 0   2 | 4

(b)   2 | 7   (f)   6 | 3
       ——|—— (observed samples)   ——|——
       5 | 1   1 | 5

(c)   3 | 6   (g)   7 | 2
       ——|——   ——|——
       4 | 2   0 | 6

(d)   4 | 5
       ——|——
       3 | 3

(2). Add together the logarithms of the factorials of the four marginal totals and subtract the logarithm of the factorial of the grand total.

		Log factorial
Marginal totals	7	3.7024
	8	4.6055
	9	5.5598
	6	2.8573
		16.7250
Grand total	15	−12.1165
		4.6085

The result can be called the 'marginal value'.

(3). Take the first of the possible pairs of samples. Find the logarithm of the factorial of each of the numbers in its four cells and add these logarithms together:

Log factorial 6   2.8573
    "   "   8   4.6055

                    7.4628

(The factorial of 1 is 1, and so is the factorial of zero. Therefore the logarithm is zero.)

Do likewise for each of the other possible pairs, and either enter each of the sums near its own pair of samples or mark them to show from which samples they are derived:

Sums of logs of factorials   Sums of logs of factorials

(a)   7.4628   (e)   5.1406
(b)   6.0826   (f)   5.7147
(c)   5.3167   (g)   6.8607
(d)   5.0158

(4). From the 'marginal value' found in (2) subtract the first of the seven quantities found in Stage (3): $4.6085 - 7.4628 = \bar{3}.1457$. Do likewise for the other six quantities in Stage (3), to give:

(a)	$\bar{3}.1457$	(e)	$\bar{1}.4679$
(b)	$\bar{2}.5259$	(f)	$\bar{2}.8938$
(c)	$\bar{1}.2918$	(g)	$\bar{3}.7478$
(d)	$\bar{1}.5927$		

(5). Convert to antilogarithms the quantities found in Stage (4). These antilogarithms are probabilities. Record each of them alongside its own pair of samples:

(a)	$\dfrac{1 \mid 8}{6 \mid 0}$	0.0014
(b)	$\dfrac{2 \mid 7}{5 \mid 1}$	0.0336 (observed samples)
(c)	$\dfrac{3 \mid 6}{4 \mid 2}$	0.1958
(d)	$\dfrac{4 \mid 5}{3 \mid 3}$	0.3914
(e)	$\dfrac{5 \mid 4}{2 \mid 4}$	0.2937
(f)	$\dfrac{6 \mid 3}{1 \mid 5}$	0.0783
(g)	$\dfrac{7 \mid 2}{0 \mid 6}$	0.0056
		0.9998 (total)

(6). Since all possible types of sample are displayed, the sum of the probabilities should be 1.0000. Test the whole calculation by adding them together. As the fourth decimal figure in each item is only an approximation, the sum often has an error, excess or defect, in the fourth place, as in this example. Values of 0.9997 or 1.0003 can be passed. Occasionally greater errors are due to the same cause, but before they are accepted it is advisable to repeat the whole calculation, sometimes with logarithms that contain seven decimal figures.

(7). P is the probability of the observed pair of samples plus the probability of rarer samples in the same tail. Therefore $P = 0.0336 + 0.0014 = 0.0350$.

Summary of Main Steps

It will be seen that the main part of the calculation can be summarized thus: From the totals find $\dfrac{6!\ 7!\ 8!\ 9!}{15!}$, and multiply it in turn by each of the quantities derived from the cells—

$$\frac{1}{6!\ 8!}, \qquad \frac{1}{2!\ 5!\ 7!}, \qquad \frac{1}{2!\ 3!\ 4!\ 6!} \quad \text{etc.}$$

Partial Computation

In the problems where the exact method is most needed it is not very laborious, for it often requires no more than half a dozen probabilities; and if we are willing to forego the benefit of the automatic check by means of the total (1.0000) we require only the probabilities from the observed samples to the end of the tail in which they lie, or until we meet a probability of 0.0000.

It is often sufficient to calculate only one term in the series, i.e., the probability of the observed pair of samples:

(1). When the observed samples are at the end of the series, i.e., contain zero in one cell.

(2). When the probability of the observed samples alone is greater than 0.025 and we desire merely a statement of significance, not the exact value of P. If the observed samples are in a long tail, therefore, it saves labor to calculate their probability first. If it is below 0.025, one must, of course, calculate also the probabilities of the rarer samples in the same tail.

Precise Assessment of Chances and Odds

Sometimes, before further investigation is carried out, it is desirable to weigh the available evidence very precisely. In Example 25 of Section B the data are:

Group	*Rh* negative	*Rh* positive	Total
(a)	6	47	53
(b)	14	42	56
Total	20	89	109

Chi square (corrected for continuity) = 2.55; $\frac{1}{2}P$ from chi square is between 0.10 and 0.05, nearer the latter. If $\frac{1}{2}P = 0.025$ is the criterion, the difference between the two groups is not significant, but the observer might say: "Although the difference does not reach the conventional standard of significance, I should like to pursue the investigation. I should, however, like to know the chances of my being led astray, i.e., of seeking for a real difference when no real difference exists. In problems like the present one, I should not pursue the investigation if the observed samples were in the opposite tail, i.e., if they differed in the opposite way from what I expected—in this instance a higher proportion of *Rh* negative in Group (a) than in Group (b)".

The problem being a one-sided comparison (Section A4; Section C, Note 5), the observer can be informed that, if he pursues investigations in which $\frac{1}{2}P$ from chi square is between 0.05 and 0.10, he will in the long run be led astray, in the sense specified above, in between 5 and 10% of his investigations. (If it were a two-sided comparison he would be led astray in from 10 to 20% of

his investigations.)   It may, however, be desirable to calculate the probability precisely.   The probabilities of the observed samples and of those that differ more, in the same direction, are:

Samples	Probability
6 : 47/14 : 42	0.0371
5 : 48/15 : 41	0.0130
4 : 49/16 : 40	0.0034
3 : 50/17 : 39	0.0006
2 : 51/18 : 38	0.0001
1 : 52/19 : 37	0.0000
0 : 53/20 : 36	0.0000
	0.0542

We can therefore replace the rough estimate, 5 to 10% error, by the precise statement—5.42%.

In this instance the exact probability has not added much to the knowledge gained from chi square; but in many cases, especially where samples are small, a verdict of nonsignificance corresponds to a surprisingly high probability, i.e., the chances of fruitless investigation are very great if the verdict is disregarded.

In Example 18 of Section B the data were:

	Recoveries	Deaths
Sulphonamide alone	1	4
Sulphonamide–penicillin	8	7

Table V shows that there is no significant difference, but does not provide a probability statement.   The exact method gives:

	Samples	Probabilities
(a)	5 : 0/4 : 11	0.0081
(b)	4 : 1/5 : 10	0.0894
(c)	3 : 2/6 : 9	0.2981
(d)	2 : 3/7 : 8	0.3832
(e)	1 : 4/8 : 7	0.1916 (observed samples)
(f)	0 : 5/9 : 6	0.0298
		1.0002

The probability, P, for (e) the observed samples and (f) the other possible rarer samples in the same tail = 0.1916 + 0.0298 = 0.2214.

The implications of this result are stated in Example 18.

NOTE 14—CHI SQUARE IN CONTINGENCY TESTS

In Example 20 of Section B the data were:

	Users of DDT	Nonusers of DDT	Total
Soldiers with scabies	(a) 29	(b) 23	52
Soldiers without scabies	(c) 64	(d) 36	100
Total	93	59	152

(The cell contents are lettered for convenience of reference.)

This is a fourfold table, and chi square (corrected for continuity) was calculated by formula and found to be 0.66. The formula is not an approximation but a condensation of the step-by-step method, to be shown here because it displays the rationale of the test, and because it is necessary for tables larger than fourfold. The steps are:

(1). Assume that treatment has no effect. Then the best estimate of the proportion of scabies patients is 52/152.

(2). On that assumption, calculate the number of scabies patients to be expected in a sample of 93, i.e., (52/152) of 93 = 31.81. This is the 'expected', 'hypothetical', or 'theoretical' value, t, corresponding to the observed or actual value, a, 29. Likewise, or by subtraction from the marginal totals, find the t values for the other three cells. (a is, of course, not to be confused with the cell letter (a).)

(3). Find the difference between a and t, indicated by $a \sim t$.

(4). Reduce each $a \sim t$ by 0.5. This is the correction for continuity (Notes 10 and 11), and is not to be used for tables larger than fourfold.

(5). Square each of the values found in (4) and divide each square by the corresponding t.

(6). Add together the items found in (5), to give chi square.

In the present example:

	(a)	(b)	(c)	(d)
Actual (a)	29	23	64	36
Theoretical (t)	31.81	20.19	61.19	38.81
$a \sim t$	2.81	2.81	2.81	2.81
$a \sim t - 0.5$	2.31	2.31	2.31	2.31
$(a \sim t - 0.5)^2/t$	5.34/31.81	5.34/20.19	5.34/61.19	5.34/38.81
	=0.17	=0.26	=0.09	=0.14

χ_c^2, i.e., chi square corrected for continuity = $0.17 + 0.26 + 0.09 + 0.14 = 0.66$.

Degrees of Freedom

The rule given in Example 20 is: number of degrees of freedom = (number of rows of cells *minus* one) $\times$ (number of columns of cells *minus* one). For a fourfold table there is therefore one degree of freedom. The rule is easy to apply without explanation, but the meaning of the term 'freedom' can perhaps be elucidated by a simple example. In a fourfold table, to calculate the t values it is sufficient to calculate t for any one cell, because the marginal totals, along with the one t value, determine what the other three t values must be, i.e., they are not independent or 'free'.

It might be asked why Table VII is not constructed according to numbers of cells; but the rule concerning degrees of freedom shows that a 12-cell table containing two rows and six columns has five degrees of freedom, whereas if it contains four rows and three columns it has six degrees of freedom. Moreover,

chi square is used for many problems besides testing contingency tables, and for all purposes the random sampling probabilities of chi square depend on the degrees of freedom as defined for each type of problem.

Probabilities from Chi Square

Table VII shows that, for one degree of freedom, a chi square of 0.66 has a probability, P, between 0.30 and 0.50.   More precisely determined from the table of Kendall and Yule (30), Appendix Table 4A, it is 0.4166.   Applying the exact (factorial) method to our present contingency table, P is found to be 0.2079.

There is no fundamental discrepancy between these two results because the exact method gives the probability for one tail only (the tail in which the observed samples lie), whereas chi square gives the probability from both tails.   We can picture the possibilities displayed as in Note 13.   Beginning with the observed samples and running in the same direction, i.e., a lower incidence of scabies in the users of DDT—29, 23; 28, 24; 27, 25; etc.—there would be one tail of the distribution.   Running from the observed samples in the opposite direction, there would first be samples in which the incidence was more and more similar in the users and nonusers of DDT; then there would be the other tail, in which there was a higher incidence of scabies in those who used DDT.

Applying the chi square test to each of the samples we should find that it was lowest in the middle, where the samples were most alike, i.e., where there was most agreement between the observations and the hypothesis, for the hypothesis is that the samples came from the same population in respect of scabies incidence.   Farther and farther in both directions chi square would increase, and chi square tables, e.g., Table VII, show simply the probability of finding chi square values greater than certain specified values.

This explains why in the chi square test we take $P = 0.05$ and 0.01 as standards, instead of 0.025 and 0.005 as in the exact method.   For fourfold tables we can halve the P from chi square to give an estimate of the probability found by the exact method.   In the present example P from chi square $= 0.4166$; $\frac{1}{2}P = 0.2083$.   The exact $P = 0.2079$.   A little discrepancy remains because asymmetry has not been corrected for, and the correction for continuity is not perfect.

As another illustration, in Example 21 of Section B, chi square $= 8.7$. $P = 0.00318$ (Yule and Kendall (30), Appendix Table 4B), i.e., $\frac{1}{2}P = 0.00159$. Exact $P = 0.00267$.   There is considerable discrepancy, but with larger samples and greater symmetry the agreement becomes closer, and the rules given in Section B, Examples 20 and 27, safeguard against serious error.

Note.—For tables larger than fourfold we use chi square probabilities directly, without dividing them.

NOTE 15—THE USE OF THE STANDARD DEVIATION, $\sqrt{Npq}$
IN COMPARISON OF SAMPLES

In Example 25 of Section B the data are:

Group	Rh negative	Rh positive	Total
(a)	6	47	53
(b)	14	42	56
Total	20	89	109

Chi square (corrected for continuity) = 2.55. P from Table VII is between 0.20 and 0.10, i.e., $\frac{1}{2}P$ is between 0.10 and 0.05. More precisely (from Appendix Table 4B of Yule and Kendall) $\frac{1}{2}P = 0.0552$. The exact $P = 0.0542$ (see Note 13). Therefore chi square gave a close approximation.

Instead of chi square many workers still use in such comparisons the standard deviation or standard error, $\sqrt{Npq}$, or its equivalent, in percentage form, $\sqrt{A\ (100 - A)/N}$ (see Note 8). If Rh negative individuals are called A—

In Group (a), percentage of A's = $6 \times 100/53 = 11.32$;

In Group (b), percentage of A's = $14 \times 100/56 = 25.00$.

The standard deviations are—

Group (a): $\sqrt{11.32 \times 88.68/53} = \sqrt{18.9408}\%$;

Group (b): $\sqrt{25 \times 75/56} = \sqrt{33.4821}\%$.

Standard error of difference = $\sqrt{\text{S.d.}^2 \text{ of Group } (a) + \text{S.d.}^2 \text{ of Group } (b)}$ = $\sqrt{18.9408 + 33.4821} = \sqrt{52.4229} = 7.24\%$.

Difference between (a) and (b) = $25.00 - 11.32 = 13.68\%$.

Difference/S.e. of difference = $13.68/7.24 = 1.89$.

If a difference is more than twice its standard error (more precisely 1.96 S.e.) it is considered significant (P is less than 0.05). The present difference has not quite reached the minimum standard, but is near enough to make one think that a few more cases might easily show a significant difference. There is therefore considerable discrepancy between this and the chi square result. P from standard error = 0.0589 (Yule and Kendall (30), Appendix Table 3); $\frac{1}{2}P = 0.0294$. P from chi square = 0.1104; $\frac{1}{2}P = 0.0552$. Exact P (corresponding to $\frac{1}{2}P$ from chi square or standard error) = 0.0542.

The chief reason for the discrepancy is that, although the standard error test is based on the normal curve, no correction is made for continuity. Such a correction can be introduced, but it complicates the calculation, and even without that complication the standard error test is arithmetically no simpler than the calculation of chi square (corrected for continuity) from the formula for fourfold tables. There are, however, more weighty reasons for preferring chi square:

(1). Although the standard error is often a good enough crude test of significance, we do not know when to cease trusting it; whereas the weaknesses of chi square have been rather thoroughly explored (see, for instance, the rules given in Section B, Example 20).

(2). Chi square values from a number of contingency tests can be combined directly (Section B, Example 31).

(3). Chi square is the test used for tables larger than fourfold.

(4). Chi square can be used in many types of problem besides contingency tests.

NOTE 16—CONFIDENCE LIMITS OF SAMPLE DIFFERENCES*

In Section B, Example 32, the users of DDT had 31.2% incidence of scabies, the nonusers had 39.0%. We assume that, in respect of incidence of scabies, there are two separate populations (see Fig. 5, in which, for convenience of drawing, the populations have been indicated as symmetrical curves).
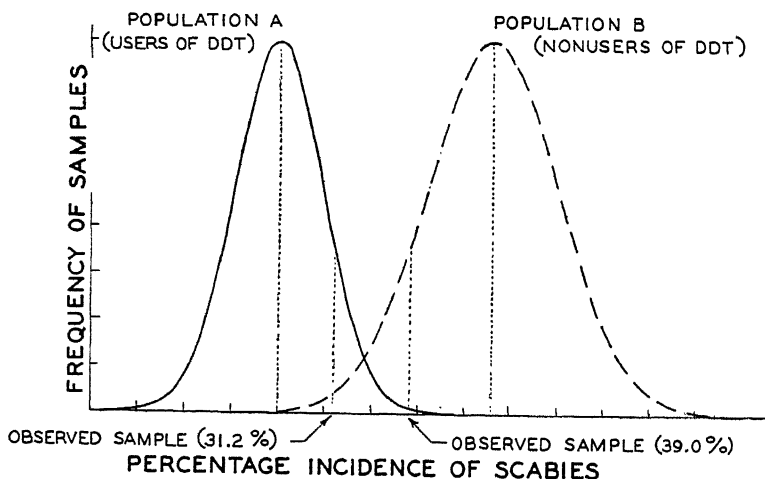
FIG. 5. *To illustrate the conception of confidence limits of sample differences.*

We estimate the true values for these populations by finding the lower confidence limit (at $P = 0.025$) from our sample of users of DDT and the upper confidence limit from our sample of those who did not use DDT. This is equivalent to pushing the two populations apart until only $2\frac{1}{2}\%$ of samples in Population A would lie to the right of the observed sample of users of DDT, i.e., $97\frac{1}{2}\%$ would lie to the left of the observed sample, and similarly $97\frac{1}{2}\%$ of samples in Population B would lie to the right of the observed sample of nonusers of DDT.

Applying the same standard in all our estimates of this kind, we shall in $97\frac{1}{2}\%$, i.e. 0.975, of our judgments, correctly state that the population value

I am indebted to Dr. J. W. Hopkins and Mr. N. Keyfitz for the solution of this problem.

for A is not higher than the one so estimated. Likewise for our estimates for Population B. Therefore in 0.975×0.975, i.e., in approximately 95%, of our combined judgments regarding the populations, we shall correctly say that the true value for A is no higher than our estimate, and that the true value for B is no lower than our estimate. Our possible error is therefore not greater than 5%—a probability of 0.05.

If a wider confidence belt is adopted ($P = 0.005$) the error is estimated similarly, i.e., by squaring 0.995 to give approximately 0.99, the probability of error being therefore 0.01 or 1%.

It should be pointed out, of course, that different confidence limits for the two populations could be chosen to give the same percentage of possible error, but leading to different estimates of the possible population differences; but there is seldom need for such elaborate calculations, departing from the confidence limits that are ordinarily used.

Note 17—Required Sample Size—No Population Difference

In Section B, Example 36, the data were:

	Died	Survived	Total
Heated	11	4	15
Cooled	4	6	10
Total	15	10	25

As a rough estimate it was shown that, even if heating and cooling did not differ in their influence on survival, probably about 1600 animals would be needed to show that the difference in survival rate in the two groups was unlikely to be more than 10%.

The numbers actually required would vary greatly, depending on what was found as the samples grew, and our original samples give little information on which to build. Since we are supposing that both samples really belong to the same population we can reasonably expect that the enlarged samples will not show a significant difference, i.e., that chi square will not exceed 3.841; but there is a wide range of possible values below that.

Detailed discussion is undesirable, but two contingency tables will illustrate some of the difficulties. They were prepared by increasing each group to 500 animals and keeping the ratio of deaths to survivals (marginal totals) at 15 : 10, i.e., 3 : 2, as in the original pair of samples. The difference between confidence limits for survival percentage was found as in Example 32.

(1)	Died	Survived	Total
Heated	306	194	500
Cooled	294	206	500
Total	600	400	1000

Chi square (corrected for continuity) = 0.50.   Difference between confidence limits = 11.1%.

(2)		Died	Survived	Total
	Heated	315	185	500
	Cooled	285	215	500
	Total	600	400	1000

Chi square (corrected for continuity) = 3.5.   Difference between confidence limits = 14.6%.

In each case larger samples would be necessary to attain the desired 10% difference between confidence limits.   According to the $\sqrt{N}$ relation (Example 36) the first table indicates the need for approximately 1200 animals, the second table indicates about 2100 animals.

All such estimates, therefore, indicate little more than orders of magnitude, and the easily computed value, 1600, is as useful as any other, in order to show an investigator whether his project is worth the effort or is possible at all in the given circumstances.

NOTE 18—CALCULATION OF P FROM THE BINOMIAL EXPANSION

Although Tables IA, IB, and II give confidence limits that are as accurate as are needed in most instances, greater accuracy may sometimes be required, and this can be obtained to any desired degree by use of the binomial expansion.   Starting with the value given in the table, we test its accuracy by calculating P.   If it is too high or too low, we shift the confidence limit slightly in the appropriate direction and again calculate P.   Several repetitions of this process will each time increase the precision of the confidence limit.

Table IB shows that if the number of A's in the sample is 19, and N is 43, the upper limit ($P = 0.025$) is 60.1%.   The estimate, expressed to two decimal places, was 60.06%, i.e., the percentage not-A was 39.94%.

Express the percentages as decimal fractions, i.e., as probabilities—p = the probability of $A = 0.6006$; q = the probability of not-A = 0.3994.   Let a = the number of A's in the sample, i.e., 19.

We require, first, to find the probability of the observed sample, and then the probabilities of rarer samples in the same tail.   The appropriate binomial formula is $(p + q)^N$, where p = the probability of A, q = the probability of not-A, and N = the number of individuals in each sample.   Then the probability of a sample that contains a individuals of Class A, is

$$\frac{N!}{(N-a)!\,a!}\,(p)^a\,(q)^{N-a}$$

Where a number of terms are required, however, it is preferable to use a modification of the formulae proposed by Gray (14).

Let $K = \log N! + N \log q$; $k = \log p - \log q$.

The logarithm of the probability of the observed sample is then:

$K + ak - \log (N - a)! - \log a!$

For successive (rarer) samples, if we are dealing with an *upper limit* we substitute for a the values $(a - 1)$, $(a - 2)$, ... If we are dealing with a *lower limit* we substitute for a the values $(a + 1)$, $(a + 2)$, ... These rules are obvious if we recall that for an upper limit the sample is in the left-hand (lower) tail of the distribution, i.e., P is the probability of occurrence of samples containing a and fewer A's, whereas for a lower limit P is the probability of a and more A's.

In the present example we proceed thus:

$$p = 0.6006; \log p = \bar{1}.7785853;$$
$$q = 0.3994; \log q = \bar{1}.6014081.$$

Seven-figure logarithms (Chambers, 23) are desirable for p and q because K and k entail the multiplying of logarithms. For the factorials, four-figure logarithms, as in Table VIII, have been found adequate.

$K = \log 43! + 43 \log 0.3994 = 52.7811 - 17.1395 = 35.6416.$

$k = \bar{1}.7785853 - \bar{1}.6014081 = - 0.2214147 + 0.3985919 = + 0.1771772.$

The first term is:

$K + 19k - \log 24! - \log 19! = 35.6416 +$
$$3.3664 - 23.7927 - 17.0851 = \bar{2}.1302.$$

The subsequent terms are:

$.K + 18k - \log 25! - \log 18!,$

$K + 17k - \log 26! - \log 17!, ...$ down to the 10th term—

$K + 10k - \log 33! - \log 10!$

To evaluate the terms, convert to antilogarithms. Thus, the first term (the probability of the observed sample) $=$ antilog $\bar{2}.1302 = 0.01350.$

The sum of the 10 probabilities is therefore:

$0.01350 + 0.00682 + 0.00314 + 0.00131 + 0.00050 + 0.00017 + 0.00005 + 0.00001 + 0.00000 + 0.00000 = 0.02550$, which is the required P.

This is higher than the desired 0.025; therefore we increase the limit (i.e., push the distribution to the right) up to 60.16%, and proceed as before. (Note that K and k are changed, but the factorials are used over again). P is now 0.02470. This is too low and linear interpolation between 60.06% ($P = 0.02550$) and 60.16% ($P = 0.02470$) gives 60.12%. This could now be tested as before, and, if desired, a closer approximation, to three or more decimal places, could similarly be found.

Useful rules for indicating the direction in which the limits require to be moved are:

Lower limit — If P is too large make the limit lower;
If P is too small make the limit higher.

Upper limit — If P is too large make the limit higher;
If P is too small make the limit lower.

Often 8 or 10 terms of the expansion are sufficient because by that time the probabilities contain five or more zeros.

When there are zero A's in a sample of N, as in Table IA, it is easy to find the upper confidence limit corresponding to any desired value of P because the first formula in this Note becomes $q^N = P$; therefore $N \log q = \log P$. Solve for q, subtract it from 1, and multiply by 100 to give the required upper limit.

NOTE 19—THE USE OF ESTIMATES AS TRUE POPULATION VALUES

This subject is discussed in Section B, Examples 1, 3, and 26. The data of Example 26 can be used to illustrate the effect. In a sample of 56 mentally defective children 14 (25%) had mothers who were Rh negative. It appeared from another author's survey that the general population contained 15% Rh negative persons, and, if this were correct, P for the observed sample of 56 would be 0.0343 (Note 8, Example 2)—the probability of chance occurrence of samples of 56 containing 14 or more Rh negative individuals.

It was found, however, that the 'population' percentage (approximately 15) was really the percentage in a sample of 334. Taking the number of Rh negatives as 50, we can compare this sample with the sample of 56 thus:

	Rh negative	Rh positive	Total
General population	50	284	334
Mentally defective	14	42	56
Total	64	326	390

Similarly, we can compare results if the sample from the general population had been 100 or 1000, with 15% Rh negative in each sample. P for chi square is determined by the table of Yule and Kendall (30), and $\frac{1}{2}P$ is taken, for comparison with P from the binomial, given above (0.0343).

Sample	Chi square	$\frac{1}{2}P$
100	1.76	0.092
334	2.82	0.047
1000	3.32	0.034

With a sample of 1000 or more, there is in this case no appreciable difference from the P value obtained by treating the sample as if it were the actual population, but, when the proportions in the two classes are more unequal, still larger samples are needed. The safe rule is to find the sample from which the so-called population frequency has been estimated and compare by a contingency table.

Note 20—Tests of Accuracy of Chi Square in Fourfold Contingency Tables

Various studies of the reliability of chi square have been made, such as those of Yates (29), Cochran (5), and Haldane (15), but they are hardly in a form suitable for direct use by most medical investigators. Table VIII of Fisher and Yates (11) provides a method of compensating for the errors of chi square in a wide range of samples; but for medical research workers it appears desirable to provide a somewhat simpler and more direct method, especially for samples not covered by the table of Fisher and Yates. Therefore our Tables IV, V, and VI were prepared, and, for samples not covered by those tables, rules for the use of chi square are presented in Example 20. These were derived partly from a rather considerable experience in comparison of chi square with the exact (factorial) test in the preparation of Tables IV, V, and VI, but largely from a series of specially designed tests.

In the special tests N_1 and N_2 were the total numbers of individuals in the respective samples. N_1 was given the values 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, and 500. For each value of N_1, N_2 was given values that were, as nearly as possible, 5, 10, 20, 40, 70, and 100% of N_1. With each value of N_2, three arrangements of A's and not-A's in N_1 were used: (1) $A = 0$; (2) $A = N_1/4$; (3) $A = N_1/2$. For arrangement (2) there were, of course, two arrangements in N_2 to be tested, according to whether the proportion of A's in N_2 was greater or less than in N_1.

Under the conditions so specified, the frequencies in N_2 were altered, step by step, to produce a minimum significant difference between the samples (P for one tail less than 0.025), and then one step farther to produce the maximum nonsignificant difference. (With some of the smaller samples, of course, only the latter was available.) For each contingency table P was found by the exact (factorial) method, and chi square (corrected for continuity) was calculated. The two results were compared by allocating the chi square value to its appropriate $\frac{1}{2}P$ interval in Table VII.

It is upon the 500 contingency tables so investigated that the rules in Example 20 are chiefly based, and it will be seen that they justify the use of chi square over a very wide range of samples, provided that no greater precision is claimed than is provided by the $\frac{1}{2}P$ intervals. A more precise estimate was attempted by interpolation in the chi square table of Yule and Kendall (30), for comparison with the exact P values. Even with the largest samples it was found that, where the distributions were markedly skew, it would be unsafe to claim greater precision than is afforded by the $\frac{1}{2}P$ intervals.

Note 21—Preparation and Accuracy of the Tables

Table IA was derived from the formula $q^N = P$—see Note 18.

Tables IB and II, Graphs 1 to 6 (= Figs. 6 to 11) were prepared from Table VIII₁ of Fisher and Yates (11). A preliminary computation of the required values with the aid of a slide rule was followed by machine computation, and discrepancies between the two sets of values were investigated. Graphs of all parts of the tables were drawn and some slight irregularities were found. These were investigated by recomputation and where this gave the same value again an estimate of its error was made by use of the binomial expansion.

A few tests of accuracy were made by application of Scheffé's (24) method to Thompson's (26) tables, and by the use of Pearson's (21) *Tables of the Incomplete Beta-function* in evaluating the terms of the binomial expansion; but it was soon found desirable to evaluate the terms directly by the method shown in Note 18. This was done not only for the values suspected of error but for other values selected from various sections of the tables, making a total of more than a hundred values so tested, in 93 of which the true values were approached by a series of steps as in Note 18.

The values, which had been originally computed with six or more figures, were then rounded off for the tables, and from the tests it would appear that the error in the tabulated values is seldom as great as ± 1 in the last decimal place.

The intervals between N values in Tables IA, IB, and II are somewhat irregular because they were chosen as the work progressed, in order to avoid too great gaps between successive values of the confidence limits without unduly increasing the amount of computation.

Table III was prepared by linear interpolation between the last two entries of Table VIII₁ of Fisher and Yates (11).

Table IV was computed by the exact method from logarithms of factorials with seven decimal figures. The probabilities were found from five-figure antilogarithms and rounded off to four decimal places for the table. Summation of the probabilities to unity gave an automatic check.

Note that P for a contingency table $O : N/O : N$ is not shown because it is unity. P values for $A : (N - A)/A : (N - A)$, where A is not zero (e.g., $2 : 13/2 : 13$), are given, and represent the probability of the observed sample plus the probabilities of samples in either tail, the distribution being symmetrical, with the observed samples at the mode.

Table V was computed by the exact method from logarithms of factorials with four decimal figures, the probabilities being checked by summation. Where the summation threw doubt on the precision, seven-figure logarithms were used. The probabilities in the table can be accepted as seldom in error by more than ± 1 in the fourth place.

Table VI—Many entries were derived from exact probabilities, computed by factorials (four-figure logarithms), the others were found by chi square, corrected for continuity and tested for significance by Table VIII of Fisher and Yates.

Note 22—Factorials

To recall the meaning of "factorial" note that factorial 4, expressed as ⌊4 or 4!, is 4 × 3 × 2 × 1. The factorial of 1 is 1, and so is the factorial of zero.

The direct use of factorials entails heavy multiplication and division; therefore logarithms of factorials are used. Table VIII contains logarithms of factorials of numbers up to 1000, the logarithms being given to four decimal places, which are sufficient for most purposes. Seven-figure logarithms are given in the tables of Fisher and Yates (11, Table XXX—numbers up to 300) and of Pearson (20, Table XLIX—numbers up to 1000).

Note 23—Random Sampling Techniques

The disk-sampling mentioned in Section A2, and described more fully in Note 1, reveals two essential features of random sampling techniques:

(1). Since the population (persons, animals, or other individuals) cannot itself be shuffled and mixed in thorough random fashion, it is represented by an artificial population that can be randomized, e.g., heads and tails on coins, spots on dice, numbers on cards or disks, or the items in a table of random numbers, such as that of Fisher and Yates (11)—the most dependable method because the numbers have been thoroughly tested for·bias.

(2). The technique must insure that each individual has an equal chance of being taken into the sample.

Two further rules should be noted:

(1). As far as possible plan the whole experiment, whether a clinical or a laboratory experiment, at the outset, including the random allocation of treatments.

(2). Having used a proper technique do not reject a sampling result because it seems nonrandom. As is well known, such extreme results occasionally occur by pure chance.

Tables of Random Numbers

The table of Fisher and Yates (six pages) contains two-figure numbers arranged in blocks to facilitate reading, for example:

26	72	39	27	67
43	00	65	98	50
16	06	10	89	20
09	65	90	77	47
65	39	07	16	29

To use the table, the observer selects anywhere in it a row, column, or diagonal, without previously inspecting the numbers themselves, and then takes the numbers in that row, column, or diagonal as they occur, passing from block to block without interruption. If only 0 to 9 are needed, only single figures are taken, e.g., the right-hand digits in the first column of the block shown above—6, 3, 6, 9, 5. If three (or more) digits are needed, one uses three (or more) adjacent columns or rows, e.g., from the first (left-hand) column and the left half of the second column in the above block—267, 430, 160, 96, 653.

Study of a few examples will enable the reader to devise methods suitable to his particular problem.

Example (1)

Patients are expected to arrive, one at a time, over a period of weeks or months. Two treatments, A and B, are to be tested on equal numbers—20 patients each.

In coin tossing let heads represent Treatment A, tails Treatment B, and let the successive tosses represent patients in order of arrival. After the first 20 heads (or tails) all Treatments A (or B) will have been allocated, and the remaining patient or patients will, of course, fall into the other class—B (or A).

In dice throwing let odd numbers represent Treatment A, even numbers Treatment B; and likewise in card dealing or disk sampling.

In a table of random numbers use one row or column, and record the odd or even digits as they occur, counting zero as even, to allow equal chances for odd and even. Thus the right-hand column in the block shown above gives 7 (odd), 0 (even), 0 (even), 7 (odd), 9 (odd).

If the individuals are already present, e.g., 40 hospital patients or animals, number them, 1 to 40, in any convenient order, e.g., by hospital beds or animal cages, and allocate treatment as above.

If the individuals are to be divided according to sex, age (adults and children), or other features, make the random allocation separately in each group.

When treatments, A and B, are to be compared it is not uncommon to apply them to alternate patients in order of arrival, or to patients in alternate beds, or animals in alternate cages—A to the first, third, fifth, and so on, B to the second, fourth, sixth, and so on. Several possibilities of bias in such an arrangement may be considered, e.g., many diseases have rhythms of severity and if, unknown to the investigator, the severity is waning and he alternates the treatment in order of the patients' arrival, the second member of each pair, receiving Treatment B, will tend on the average to have a milder attack than the first, who received Treatment A. Alternate animal cages likewise may differ in exposure to air currents, temperature, or light.

These various possibilities may be of little or no consequence, or the investigator may try in various ways to compensate for factors that he thinks may introduce bias, but the only way to guard against *hidden (unsuspected) bias*, and thereby avoid present and future doubt, is strictly random sampling. When an investigator employs a nonrandom method as if equivalent to a random technique, the onus is on him to prove it justifiable, and this would entail an additional, usually large, investigation.

Example (*2*)

If more than two treatments are to be tested, coin tossing is rather complicated. Dice throwing can be used, e.g., for three treatments, let Treatment *A* be represented by one spot and six spots, *B* by two and five, *C* by three and four. In using a set of randomly distributed items, such as numbers from cards, disks, or a table, each treatment must be represented by an equal number of items, the others being disregarded when they turn up; e.g., in allocating six treatments by sampling from 100 numbered cards or random numbers, disregard Nos. 97, 98, 99, 100 (00 in a table of random numbers) because each treatment will then be represented by 16 numbers.

Treatments *A*, *B*, *C*, *D*, *E*, and *F* are each to be tested on five animals. The 30 animals are numbered in any convenient order, and let us suppose that the random numbers in a row or column (or from cards or disks) are, in order: 03, 92, 18, 27, 00, 46, 57, 99, 16, 96, 56, 30, 33, 72, 85, 22, 84, 64, . . . A simple method, which is not unduly wasteful of numbers, is to note what remainder would be left if each number were divided by 6 (the number of treatments). Let the, remainders represent treatments: $1 = A$, $2 = B$, $3 = C$, $4 = D$, $5 = E$, $0 = F$. By this method the above random numbers would allot treatments as follows:

Random number	Remainder	Treatment	Serial No. of animal
03	3	*C*	1
92	2	*B*	2
18	0	*F*	3
27	3	*C*	4
00	Omit	—	—
46	4	*D*	5
57	3	*C*	6
99	Omit	—	—
16	4	*D*	7
96	0	*F*	8

and so on. As soon as any treatment-group has received five animals, remainders indicating that treatment are, of course, disregarded.

Example (*3*)

Five animals from a numbered set of 40 are to receive Treatment *A*, the remaining 35 are to be used as untreated controls, or to receive Treatment *B*. In a row or column of two-digit random numbers neglect 00 and all entries above 40. If, for example, the row contains 26, 72, 39, 27, 67, 90, 29, 16, animals bearing the serial numbers 26, 39, 27, 29, and 16 would receive Treatment *A* because they have been met first in the search.

Example (4)

From each of 15 litters three animals are used, to compare Treatments *A*, *B*, and *C*. In single-digit random numbers neglect zeros and allot the treatments according to remainders after dividing by 3, as in Example (2), thus:

Random number	3	2	8	7	0	6	7	9	6	6	0	3	2	1	2
Remainder	0	2	2	1	—	0	1	0	0	0	—	0	2	1	2
Treatment	*C*	*B*	*B*	*A*	—	*C*	*A*	*C*	*C*	*C*	—	*C*	*B*	*A*	*B*
Sample No.	(1)	(1)	(2)	(2)	—	(3)	(3)	(4)	—	—	—	—	(4)	(5)	(5)

In Sample (1) animal No. 1 receives *C*, animal No. 2 receives *B*. Therefore the remaining animal, No. 3, must receive *A*. In Sample (2) animal No. 1 receives *B* (the third letter), animal No. 2 receives *A*; therefore animal No. 3 receives *C*. In Sample (3) the treatments are *C*, *A*, and therefore *B*. In Sample (4) animal No. 1 receives *C*, and we must omit the three succeeding *C*'s, allocating *B* to animal No. 2, and so on.

If the samples are of twins, pairs of animals or parallel pairs of bacterial culture plates, the procedure is similar but simpler, for even numbers can represent *A* and odd numbers *B*, zero being counted as even.

Example (5)

Let us suppose that there are 234 animals in stock and that we require a random sample of 40. Running down a random number column we could take the first 40 numbers between 001 and 234, but this would waste numbers, and we can assign four random numbers to each animal, using numbers up to 4 × 234 (i.e., 936). Thus, animal No. 1 would be represented by 001, 002, 003, 004; animal No. 2 would receive the next four, and animal No. 234 would receive 933, 934, 935, 936. We should then take the first 40 numbers, in a column or row, that fell within this range, disregarding numbers outside the range, among which would be included 000 (equivalent to 1000). When an animal had already been selected, if one of its numbers appeared again it would be disregarded.

Note 24—Recommendations Regarding Mathematical Tables and Other Sources of Information

Tables. For nearly everyone who applies statistical tests to medical or biological data the tables of Fisher and Yates (11) should be considered indispensable.

Among *four-figure logarithm tables* such as are supplied to colleges, time is saved by those that contain antilogarithms, as do those of Bottomley (2). Where four figures are not accurate enough, *five-figure tables* with antilogarithms (Castle, 3) will often be sufficient and are much easier to work with than *seven-figure* tables. Sometimes, however, the latter are needed (Chambers, 23).

Many small books of tables contain *squares*, *square roots*, and *reciprocals*, but an investigator frequently finds the need for greater accuracy than they provide, and Barlow's tables (6) save much time and effort.

Graphs. Those who are using *chi square* for contingency tables and other purposes will find useful the graphs prepared by Bliss (1), Yule and Kendall (30).

Information on Methods. Although this Note is not a study guide, a little help may be desirable. Fisher's *Statistical Methods for Research Workers* (9) and *The Design of Experiments* (10) are, of course, basic works for investigators in any biological field. As regards qualitative statistics in particular, *Statistical Methods* shows the application of chi square to more complicated problems than have been discussed in this article, and it discusses the Poisson distribution. *The Design of Experiments* introduces the reader to the use of random sampling in biological experiments.

The study of literature on confidence limits could profitably begin with the article by Clopper and Pearson (4) and the one by Stevens (25) in which appeared a preliminary version of Table VIII1 of Fisher and Yates.

Acknowledgments

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* *This article is not only a valuable warning against the dangers of percentages but a source of examples from a wide variety of medical investigations.   Some have been used in Section B for the application of more accurate methods than those available when the article was written.*

Index of Subjects

References are to pages and Examples (Ex.)

TABLE IA

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF A's IN SAMPLE $= 0$

N\P	Upper limits (%) .10	.025	.005	N\P	Upper limits (%) .10	.025	.005
1	90.0	97.5	99.5	35	6.4	10.0	14.0
2	68.4	84.2	92.9	40	5.6	8.8	12.4
3	53.6	70.8	82.9	45	5.0	7.9	11.1
4	43.8	60.2	73.4	50	4.5	7.1	10.0
5	36.9	52.2	65.3	55	4.1	6.5	9.2
6	31.9	45.9	58.6	60	3.8	6.0	8.5
7	28.0	41.0	53.1	65	3.5	5.5	7.8
8	25.0	36.9	48.4	70	3.2	5.1	7.3
9	22.6	33.6	44.5	75	3.0	4.8	6.8
10	20.6	30.9	41.1	80	2.8	4.5	6.4
11	18.9	28.5	38.2	85	2.7	4.2	6.0
12	17.5	26.5	35.7	90	2.5	4.0	5.7
13	16.2	24.7	33.5	95	2.4	3.8	5.4
14	15.2	23.2	31.5	100	2.3	3.6	5.2
15	14.2	21.8	29.8	110	2.1	3.3	4.7
16	13.4	20.6	28.2	120	1.9	3.0	4.3
17	12.7	19.5	26.8	130	1.8	2.8	4.0
18	12.0	18.5	25.5	140	1.6	2.6	3.7
19	11.4	17.6	24.3	150	1.5	2.4	3.5
20	10.9	16.8	23.3	160	1.4	2.3	3.3
21	10.4	16.1	22.3	170	1.3	2.1	3.1
22	9.9	15.4	21.4	180	1.3	2.0	2.9
23	9.5	14.8	20.6	190	1.2	1.9	2.7
24	9.2	14.3	19.8	200	1.1	1.8	2.6
25	8.8	13.7	19.1	220	1.0	1.7	2.4
26	8.5	13.2	18.4	250	.92	1.5	2.1
27	8.2	12.8	17.8	300	.76	1.2	1.8
28	7.9	12.3	17.2	400	.57	.92	1.3
29	7.6	11.9	16.7	500	.46	.74	1.1
30	7.4	11.6	16.2	700	.32	.53	.76
				1000	.23	.37	.53

*Note.—For mode of use see Example 7. N = total number of individuals in the sample.
P = probability.   For $N > 1000$, divide values for $N = 1000$ by $N/1000$.   To find limits corresponding to other values of P, see the last paragraph of Note 18 in Section C.*

TABLE IB

Confidence Limits for Twofold Classification of Enumeration Data—Number of *A*'s in Sample: 1 to 20

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 1

N\P	.005	.025	.10	.10	.025	.005
2	.25	1.3	5.2	94.8	98.7	99.75
3	.17	.84	3.5	80.4	90.6	95.9
4	.12	.63	2.6	68.0	80.6	88.9
5	.10	.51	2.1	58.4	71.6	81.4
6	.083	.42	1.7	51.2	64.2	74.7
7	.071	.36	1.5	45.3	57.9	68.6
8	.063	.32	1.3	40.6	52.8	63.2
9	.056	.28	1.2	36.9	48.3	58.6
10	.050	.25	1.0	33.7	44.5	54.4
11	.045	.23	.95	31.1	41.3	50.9
12	.042	.21	.88	28.7	38.5	47.8
13	.039	.19	.81	26.8	36.1	45.1
14	.036	.18	.75	25.1	33.9	42.5
15	.033	.17	.70	23.6	32.0	40.3
16	.031	.16	.66	22.2	30.2	38.2
17	.029	.15	.62	21.1	28.7	36.4
18	.028	.14	.58	19.9	27.3	34.7
19	.026	.13	.55	19.0	26.0	33.1
20	.025	.13	.52	18.1	24.8	31.7
21	.024	.12	.50	17.3	23.8	30.4
22	.023	.12	.48	16.5	22.8	29.3
23	.022	.11	.46	15.9	22.0	28.2
24	.021	.11	.44	15.3	21.1	27.2
25	.020	.10	.42	14.7	20.4	26.2
26	.019	.097	.40	14.2	19.7	25.3
27	.018	.094	.39	13.7	19.0	24.5
28	.018	.090	.38	13.2	18.4	23.7
29	.017	.087	.36	12.8	17.8	23.0
30	.017	.084	.35	12.4	17.2	22.3
32	.016	.079	.33	11.6	16.2	21.1
35	.014	.072	.30	10.7	14.9	19.5
40	.013	.063	.26	9.4	13.2	17.2
45	.011	.056	.23	8.4	11.8	15.4
50	.010	.051	.21	7.6	10.7	14.0
60	.0084	.042	.18	6.3	9.0	11.8
70	.0071	.036	.15	5.4	7.7	10.2
80	.0062	.032	.13	4.8	6.8	9.0
90	.0056	.028	.12	4.3	6.0	8.0
100	.0050	.025	.11	3.8	5.4	7.2
110	.0045	.023	.095	3.5	5.0	6.6
120	.0042	.021	.088	3.2	4.6	6.0
150	.0033	.017	.070	2.6	3.7	4.9
160	.0031	.016	.066	2.4	3.4	4.6
200	.0025	.013	.053	1.9	2.8	3.7
300	.0017	.0084	.035	1.3	1.8	2.5
500	.0010	.0051	.021	.78	1.1	1.5
1000	.00050	.0025	.011	.39	.56	.74

Note.—For mode of use see Examples 1 to 7. *N* = total number of individuals in sample. *P* = probability. For *N* > 1000, divide values for *N* = 1000 by *N*/1000.

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 2

N	.005	.025	.10	.10	.025	.005
4	3.0	6.8	14.2	85.8	93.2	97.0
5	2.3	5.3	11.2	75.4	85.3	91.7
6	1.9	4.3	9.3	66.7	77.8	85.6
7	1.6	3.7	7.9	59.6	71.0	79.1
8	1.4	3.2	6.9	53.9	65.1	74.2
9	1.2	2.8	6.1	49.1	60.0	69.3
10	1.1	2.5	5.4	45.0	55.6	64.8
11	.98	2.3	4.9	41.5	51.8	60.9
12	.89	2.1	4.5	38.6	48.4	57.4
13	.82	1.9	4.2	36.0	45.5	54.2
14	.76	1.8	3.9	33.7	42.8	51.3
15	.71	1.7	3.6	31.7	40.5	48.7
16	.67	1.6	3.4	30.0	38.4	46.3
17	.63	1.5	3.2	28.4	36.5	44.2
18	.59	1.4	3.0	27.0	34.7	42.2
19	.56	1.3	2.8	25.7	33.1	40.4
20	.53	1.2	2.7	24.5	31.7	38.7
21	.50	1.2	2.6	23.4	30.4	37.2
22	.48	1.1	2.4	22.4	29.2	35.8
23	.46	1.1	2.3	21.5	28.1	34.4
24	.44	1.0	2.2	20.7	27.0	33.2
25	.42	.98	2.1	19.9	26.0	32.1
26	.41	.95	2.1	19.2	25.1	31.0
27	.39	.91	2.0	18.6	24.3	30.0
28	.38	.88	1.9	17.9	23.5	29.1
29	.36	.85	1.8	17.3	22.8	28.2
30	.35	.82	1.8	16.8	22.1	27.4
32	.33	.77	1.7	15.8	20.8	25.8
35	.30	.70	1.5	14.5	19.2	23.8
40	.26	.61	1.3	12.8	16.9	21.1
45	.23	.54	1.2	11.4	15.2	19.0
50	.21	.49	1.1	10.3	13.7	17.2
60	.17	.41	.89	8.6	11.5	14.5
70	.15	.35	.76	7.4	10.0	12.6
80	.13	.30	.67	6.5	8.7	11.1
90	.12	.27	.59	5.8	7.8	9.9
100	.10	.24	.53	5.2	7.0	8.9
120	.086	.20	.44	4.4	5.9	7.5
150	.069	.16	.36	3.5	4.7	6.0
200	.052	.12	.27	2.6	3.6	4.6
300	.034	.081	.18	1.8	2.4	3.1
500	.021	.048	.11	1.1	1.4	1.8
1000	.010	.024	.053	.53	.72	.92

Note.—For mode of use see Examples 1 to 7. N = total number of individuals in sample.
P = probability. For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 3

N	.005	.025	.10	.10	.025	.005
6	6.6	11.8	20.2	79.8	88.2	93.4
7	5.5	9.9	17.0	72.1	81.6	88.3
8	4.8	8.5	14.6	65.5	75.5	83.0
9	4.2	7.5	13.0	59.9	70.1	78.1
10	3.7	6.7	11.6	55.2	65.2	73.5
11	3.3	6.0	10.5	51.1	61.0	69.4
12	3.0	5.5	9.6	47.5	57.2	65.7
13	2.8	5.0	8.8	44.4	53.8	62.2
14	2.6	4.7	8.2	41.7	50.8	59.0
15	2.4	4.3	7.6	39.3	48.1	56.1
16	2.2	4.0	7.1	37.1	45.6	53.5
17	2.1	3.8	6.7	35.2	43.4	51.1
18	2.0	3.6	6.3	33.4	41.4	48.9
19	1.9	3.4	6.0	31.9	39.6	46.9
20	1.8	3.2	5.6	30.4	37.9	45.0
21	1.7	3.1	5.4	29.1	36.4	43.3
22	1.6	2.9	5.1	27.9	34.9	41.7
23	1.5	2.8	4.9	26.8	33.6	40.2
24	1.5	2.7	4.7	25.8	32.4	38.8
25	1.4	2.5	4.5	24.8	31.2	37.5
26	1.3	2.4	4.3	23.9	30.2	36.2
27	1.3	2.4	4.2	23.1	29.2	35.1
28	1.2	2.3	4.0	22.3	28.2	34.0
29.	1.2	2.2	3.9	21.6	27.4	33.0
30	1.2	2.1	3.7	20.9	26.5	32.0
32	1.1	2.0	3.5	19.7	25.0	30.3
35	.99	1.8	3.2	18.1	23.1	28.0
40	.86	1.6	2.8	16.0	20.4	24.9
45	.77	1.4	2.5	14.2	18.3	22.4
50	.69	1.3	2.2	12.9	16.6	20.3
60	.57	1.0	1.9	10.8	13.9	17.2
70	.49	.89	1.6	9.3	12.0	14.9
80	.43	.78	1.4	8.2	10.6	13.1
90	.38	.69	1.2	7.3	9.4	11.7
100	.34	.62	1.1	6.6	8.5	10.6
120	.28	.52	.92	5.5	7.1	8.9
150	·23	·41	·74	4.4	5·7	7·1
200	.17	.31	.55	3.3	4.3	5.4
300	.11	.21	.37	2.2	2.9	3.6
500	.068	.12	.22	1.3	1.7	2.2
1000	.034	.062	.11	.67	.87	1.1

*Note.—For mode of use see Examples 1 to 7.   N = total number of individuals in sample.
P = probability.   For N > 1000, divide values for N = 1000 by N/1000.*

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 4

8	10.0	15.8	24.0	76.0	84.2	90.0
9	8.7	13.8	21.1	69.9	78.8	85.5
10	7.7	12.2	18.8	64.6	73.8	80.9
11	6.9	11.0	17.0	60.0	69.3	76.8
12	6.2	9.9	15.4	55.9	65.2	72.8
13	5.7	9.1	14.2	52.3	61.5	69.1
14	5.3	8.4	13.1	49.2	58.1	65.8
15	4.9	7.8	12.2	46.4	55.1	62.8
16	4.6	7.3	11.4	43.9	52.4	60.0
17	4.3	6.8	10.7	41.6	49.9	57.4
18	4.0	6.4	10.1	39.6	47.7	55.0
19	3.8	6.1	9.5	37.7	45.5	52.7
20	3.6	5.8	9.0	36.0	43.6	50.6
21	3.4	5.5	8.6	34.5	41.9	48.8
22	3.2	5.2	8.2	33.1	40.3	47.0
23	3.1	5.0	7.8	31.8	38.8	45.4
24	3.0	4.8	7.5	30.6	37.4	43.8
25	2.8	4.6	7.2	29.5	36.1	42.4
26	2.7	4.4	6.9	28.4	34.9	41.1
27	2.6	4.2	6.6	27.5	33.8	39.8
28	2.5	4.0	6.4	26.5	32.7	38.6
29	2.4	3.9	6.1	25.7	31.6	37.4
30	2.3	3.8	5.9	24.9	30.7	36.4
32	2.2	3.5	5.6	23.4	29.0	34.4
35	2.0	3.2	5.1	21.6	26.8	31.8
37	1.9	3.0	4.8	20.5	25.4	30.3
40	1.7	2.8	4.4	19.0	23.7	28.3
45	1.5	2.5	3.9	17.0	21.2	25.4
50	1.4	2.2	3.5	15.4	19.2	23.1
55	1.3	2.0	3.2	14.0	17.6	21.2
60	1.1	1.9	2.9	12.9	16.2	19.6
70	.98	1.6	2.5	11.1	14.0	16.9
80	.85	1.4	2.2	9.7	12.3	14.9
90	.76	1.2	1.9	8.7	11.0	13.4
100	.68	1.1	1.8	7.8	9.9	12.1
120	.57	.92	1.5	6.5	8.2	10.1
150	.45	.73	1.2	5.3	6.7	8.2
200	.34	.55	.87	4.0	5.0	6.2
300	.22	.36	.58	2.6	3.4	4.1
500	.13	.22	.35	1.6	2.0	2.5
1000	.067	.11	.17	.80	1.0	1.3

Note.—*For mode of use see Examples 1 to 7.   N = total number of individuals in sample.
P = probability.   For N > 1000, divide values for N = 1000 by N/1000.*

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N＼P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample

10	12.8	18.7	26.7	73.3	81.3	87.2
11	11.4	16.8	24.0	68.2	76.6	83.2
12	10.3	15.2	21.8	63.8	72.3	79.2
13	9.4	13.9	20.0	59.8	68.4	75.5
14	8.7	12.8	18.5	56.3	64.9	72.1
15	8.0	- 11.8	17.2	53.2	61.6	68.9
16	7.5	11.0	16.0	50.3	58.7	65.9
17	7.0	10.3	15.0	47.8	55.9	63.1
18	6.6	9.7	14.2	45.5	53.5	60.6
19	6.2	9.2	13.4	43.4	51.2	58.3
20	5.8	8.7	12.7	41.5	49.1	56.0
21	5.5	8.2	12.1	39.7	47.2	54.0
22	5.3	7.8	11.5	38.1	45.4	52.1
23	5.0	7.5	11.0	36.6	43.7	50.3
24	4.8	7.1	10.5	35.2	42.2	48.6
25	4.6	6.8	10.1	34.0	40.7	47.0
26	4.4	6.6	9.7	32.8	39.4	45.5
27	4.2	6.3	9.3	31.7	38.1	44.2
28	4.1	6.1	9.0	30.6	36.9	42.8
29	3.9	5.8	8.6	29.6	35.8	41.6
30	3.8	5.6	8.3	28.7	34.7	40.4
32	3.5	5.3	7.8	27.1	32.8	38.3
35	3.2	4.8	7.1	24.9	30.3	35.4
37	3.0	4.5	6.7	23.6	28.8	33.8
40	2.8	4.2	6.2	22.0	26.8	31.5
42	2.7	4.0	5.9	21.0	25.6	30.2
45	2.5	3.7	5.5	19.6	24.1	28.4
50	2.2	3.3	4.9	17.8	21.8	25.8
55	2.0	3.0	4.5	16.2	20.0	23.7
60	1.8	2.8	4.1	14.9	18.4	21.9
70	1.6	2.4	3.5	12.8	15.9	18.9
80	1.4	2.1	3.1	11.3	14.0	16.7
90	1.2	1.8	2.7	10.1	12.5	14.9
100	1.1	1.6	2.4	9.1	11.3	13.5
120	.91	1.4	2.0	7.6	9.5	11.4
150	.73	1.1	1.6	6.1	7.6	9.2
200	.54	.82	1.2	4.6	5.7	6.9
300	.36	.54	.81	3.1	3.8	4.6
500	.22	.32	.49	1.8	2.3	2.8
1000	.11	.16	.24	.92	1.2	1.4

Note.—For mode of use see Examples 1 to 7.   N = total number of individuals in sample. P = probability.   For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N＼P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 6

N＼P	.005	.025	.10	.10	.025	.005
12	15.2	21.1	28.8	71.2	78.9	84.7
13	13.8	19.2	26.4	66.9	74.9	81.2
14	12.7	17.7	24.3	63.1	71.2	77.7
15	11.7	16.3	22.5	59.7	67.7	74.4
16	10.8	15.2	21.0	56.6	64.6	71.4
17	10.1	14.2	19.7	53.7	61.7	68.5
18	9.5	13.4	18.6	51.2	59.0	65.8
19	8.9	12.6	17.5	48.9	56.6	63.3
20	8.4	11.9	16.6	46.8	54.3	61.0
21	8.0	11.3	15.8	44.8	52.2	58.8
22	7.6	10.7	15.0	43.0	50.2	56.8
23	7.2	10.2	14.3	41.3	48.4	54.9
24	6.9	9.8	13.7	39.8	46.7	53.1
25	6.6	9.4	13.1	38.3	45.1	51.4
26	6.3	9.0	12.6	37.0	43.7	49.8
27	6.1	8.6	12.1	35.8	42.3	48.3
28	5.9	8.3	11.7	34.6	41.0	46.9
29	5.6	8.0	11.3	33.5	39.7	45.5
30	5.4	7.7	10.9	32.5	38.6	44.3
32	5.1	7.2	10.2	30.6	36.4	42.0
35	4.6	6.6	9.3	28.2	33.7	38.9
37	4.4	6.2	8.7	26.7	32.0	37.1
40	4.0	5.7	8.1	24.8	29.8	34.6
42	3.8	5.4	7.7	23.7	28.5	33.2
45	3.5	5.1	7.2	22.2	26.8	31.2
50	3.2	4.5	6.4	20.1	24.3	28.4
55	2.9	4.1	5.8	18.4	22.2	26.1
60	2.6	3.8	5.3	16.9	20.5	24.1
65	2.4	3.5	4.9	15.6	19.0	22.4
70	2.2	3.2	4.6	14.6	17.7	20.9
80	2.0	2.8	4.0	12.8	15.6	18.4
90	1.7	2.5	3.5	11.4	13.9	16.5
100	1.6	2.2	3.2	10.3	12.6	14.9
120	1.3	1.9	2.6	8.6	10.6	12.5
150	1.0	1.5	2.1	6.9	8.5	10.1
200	.78	1.1	1.6	5.2	6.4	7.6
300	.52	.74	1.1	3.5	4.3	5.1
500	.31	.44	.63	2.1	2.6	3.1
1000	.15	.22	.32	1.1	1.3	1.6

Note.—*For mode of use see Examples 1 to 7. N = total number of individuals in sample.*
P = probability. For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N \ P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 7

N						
14	17.2	23.1	30.4	69.6	76.9	82.8
15	15.9	21.3	28.2	65.8	73.4	79.5
16	14.7	19.8	26.3	62.5	70.1	76.4
17	13.7	18.4	24.6	59.4	67.0	73.5
18	12.8	17.3	23.1	56.6	64.2	70.7
19	12.1	16.3	21.8	54.1	61.6	68.1
20	11.4	15.4	20.6	51.8	59.2	65.7
21	10.8	14.6	19.6	49.6	57.0	63.4
22	10.2	13.8	18.7	47.7	54.9	61.3
23	9.8	13.2	17.8	45.8	52.9	59.2
24	9.3	12.6	17.0	44.1	51.1	57.3
25	8.9	12.1	16.3	42.6	49.4	55.6
26	8.5	11.6	15.6	41.1	47.8	53.9
27	8.2	11.1	15.0	39.7	46.3	52.3
28	7.9	10.7	14.5	38.4	44.9	50.8
29	7.6	10.3	14.0	37.2	43.5	49.4
30	7.3	9.9	13.5	36.1	42.3	48.0
31	7.0	9.6	13.0	35.0	41.1	46.7
32	6.8	9.3	12.6	34.0	40.0	45.5
33	6.6	9.0	12.2	33.1	38.9	44.4
34	6.4	8.7	11.8	32.2	37.9	43.2
35	6.2	8.4	11.5	31.3	36.9	42.2
37	5.8	8.0	10.8	29.8	35.2	40.3
40	5.4	7.3	10.0	27.7	32.8	37.6
42	5.1	7.0	9.5	26.4	31.4	36.1
45	4.7	6.5	8.9	24.8	29.5	34.0
47	4.5	6.2	8.5	23.8	28.3	32.7
50	4.2	5.8	8.0	22.4	26.7	30.9
55	3.8	5.3	7.2	20.5	24.5	28.4
60	3.5	4.8	6.6	18.8	22.6	26.2
65	3.2	4.4	6.1	17.4	20.9	24.3
70	3.0	4.1	5.7	16.2	19.5	22.7
80	2.6	3.6	4.9	14.3	17.2	20.1
90	2.3	3.2	4.4	12.7	15.4	18.0
100	2.1	2.9	3.9	11.5	13.9	16.3
120	1.7	2.4	3.3	9.6	11.6	13.7
150	1.4	1.9	2.6	7.7	9.4	11.0
200	1.0	1.4	2.0	5.8	7.1	8.4
300	.68	.94	1.3	3.9	4.7	5.6
500	.41	.56	.78	2.3	2.9	3.4
1000	.20	.28	.39	1.2	1.4	1.7

Note.—For mode of use see Examples 1 to 7. N = total number of individuals in sample. P = probability. For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 8

N\P	.005	.025	.10	.10	.025	.005
16	19.0	24.6	31.8	68.2	75.4	81.0
17	17.7	23.0	29.8	64.9	72.2	78.1
18	16.5	21.5	27.9	62.0	69.3	75.3
19	15.5	20.2	26.3	59.2	66.5	72.6
20	14.6	19.1	24.9	56.8	64.0	70.1
21	13.8	18.1	23.6	54.4	61.6	67.7
22	13.1	17.2	22.5	52.3	59.3	65.5
23	12.5	16.4	21.4	50.3	57.3	63.4
24	11.9	15.6	20.5	48.5	55.3	61.4
25	11.4	15.0	19.6	46.7	53.5	59.5
26	10.9	14.3	18.8	45.1	51.8	57.8
27	10.4	13.8	18.1	43.7	50.2	56.1
28	10.0	13.2	17.4	42.2	48.7	54.5
29	9.6	12.7	16.8	40.9	47.2	53.0
30	9.3	12.3	16.2	39.7	45.9	51.6
31	9.0	11.9	15.7	38.5	44.6	50.2
32	8.7	11.5	15.1	37.4	43.4	48.9
33	8.4	11.1	14.7	36.4	42.3	47.7
34	8.1	10.7	14.2	35.4	41.2	46.5
35	7.9	10.4	13.8	34.5	40.1	45.4
37	7.4	9.8	13.0	32.7	38.2	43.4
40	6.8	9.0	12.0	30.4	35.6	40.6
42	6.5	8.6	11.4	29.1	34.1	38.9
45	6.0	8.0	10.6	27.3	32.1	36.6
47	5.8	7.6	10.2	26.2	30.8	35.2
50	5.4	7.2	9.5	24.7	29.1	33.4
55	4.9	6.5	8.7	22.6	26.7	30.6
60	4.5	5.9	7.9	20.8	24.6	28.3
65	4.1	5.5	7.3	19.2	22.8	26.3
70	3.8	5.1	6.8	17.9	21.3	24.6
80	3.3	4.4	5.9	15.7	18.8	21.7
90	2.9	3.9	5.2	14.0	16.8	19.4
100	2.6	3.5	4.7	12.7	15.2	17.6
120	2.2	2.9	3.9	10.6	12.7	14.8
150	1.7	2.3	3.1	8.5	10.2	12.0
200	1.3	1.7	2.3	6.4	7.7	9.0
300	.86	1.2	1.6	4.3	5.2	6.1
500	.52	.69	.93	2.6	3.1	3.7
1000	.26	.35	.47	1.3	1.6	1.8

Note.—*For mode of use see Examples 1 to 7. N = total number of individuals in sample.
= probability. For N > 1000, divide values for N = 1000 by N/1000.*

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 9

N\P	.005	.025	.10	.10	.025	.005
18	20.4	26.0	32.9	67.1	74.0	79.6
19	19.2	24.4	31.0	64.2	71.1	76.9
20	18.1	23.0	29.3	61.5	68.5	74.3
21	17.1	21.8	27.8	59.0	66.0	71.9
22	16.2	20.7	26.4	56.7	63.6	69.5
23	15.4	19.7	25.2	54.6	61.5	67.4
24	14.7	18.8	24.1	52.6	59.4	65.3
25	14.0	18.0	23.0	50.8	57.5	63.4
26	13.4	17.2	22.1	49.1	55.7	61.5
27	12.9	16.5	21.2	47.5	54.0	59.8
28	12.3	15.9	20.4	45.9	52.3	58.1
29	11.9	15.3	19.7	44.5	50.8	56.5
30	11.4	14.7	19.0	43.2	49.4	55.0
31	11.0	14.2	18.4	41.9	48.0	53.6
32	10.7	13.7	17.8	40.7	46.8	52.2
33	10.3	13.3	17.2	39.6	45.5	50.9
34	10.0	12.9	16.7	38.6	44.4	49.7
35	9.7	12.5	16.2	37.5	43.3	48.5
37	9.1	11.8	15.3	35.7	41.2	46.3
40	8.4	10.8	14.1	33.2	38.5	43.4
42	7.9	10.3	13.4	31.7	36.8	41.6
45	7.4	9.6	12.4	29.7	34.6	39.2
47	7.0	9.2	11.9	28.5	33.3	37.7
50	6.6	8.6	11.2	26.9	31.4	35.7
55	6.0	7.8	10.1	24.6	28.8	32.8
60	5.5	7.1	9.3	22.6	26.6	30.4
65	5.0	6.5	8.5	21.0	24.7	28.2
70	4.6	6.1	7.9	19.5	23.0	26.4
80	4.0	5.3	6.9	17.2	20.3	23.3
90	3.6	4.7	6.1	15.3	18.1	20.9
100	3.2	4.2	5.5	13.8	16.4	18.9
120	2.7	3.5	4.6	11.6	13.8	15.9
150	2.1	2.8	3.7	9.3	11.1	12.9
200	1.6	2.1	2.7	7.0	8.4	9.7
250	1.3	1.7	2.2	5.6	6.7	7.8
300	1.1	1.4	1.8	4.7	5.6	6.5
500	.63	.83	1.1	2.8	3.4	4.0
1000	.31	.41	.54	1.4	1.7	2.0

*Note.—For mode of use see Examples 1 to 7. N = total number of individuals in samp
= probability. For N > 1000, divide values for N = 1000 by N/1000.*

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 10

20	21.8	27.2	33.8	66.2	72.8	78.2
21	20.5	25.7	32.0	63.6	70.2	75.8
22	19.5	24.4	30.5	61.1	67.8	73.4
23	18.5	23.2	29.0	58.8	65.5	71.2
24	17.6	22.1	27.7	56.7	63.3	69.1
25	16.8	21.1	26.5	54.8	61.3	67.0
26	16.1	20.2	25.4	52.9	59.4	65.1
27	15.4	19.4	24.4	51.2	57.6	63.3
28	14.8	18.6	23.5	49.6	55.9	61.6
29	14.2	17.9	22.6	48.0	54.3	59.9
30	13.7	17.3	21.8	46.6	52.8	58.4
31	13.2	16.7	21.1	45.3	51.4	56.9
32	12.7	16.1	20.4	44.0	50.0	55.4
33	12.3	15.6	19.7	42.8	48.7	54.1
34	11.9	15.1	19.1	41.6	47.5	52.8
35	11.5	14.6	18.6	40.5	46.3	51.5
37	10.9	13.8	17.5	38.5	44.1	49.2
40	10.0	12.7	16.2	35.9	41.2	46.1
42	9.5	12.1	15.4	34.3	39.5	44.3
45	8.8	11.2	14.3	32.1	37.1	41.7
47	8.4	10.7	13.7	30.9	35.7	40.2
50	7.9	10.0	12.8	29.1	33.7	38.0
52	7.5	9.6	12.3	28.1	32.5	36.8
55	7.1	9.1	11.6	26.6	30.9	35.0
60	6.5	8.3	10.6	24.5	28.5	32.4
65	6.0	7.6	9.8	22.7	26.5	30.1
70	5.5	7.1	9.1	21.2	24.7	28.1
75	5.1	6.6	8.5	19.8	23.2	26.4
80	4.8	6.2	7.9	18.6	21.8	24.9
90	4.3	5.5	7.0	16.6	19.5	22.3
100	3.8	4.9	6.3	15.0	17.6	20.2
110	3.5	4.4	5.7	13.7	16.1	18.5
120	3.2	4.1	5.2	12.5	14.8	17.0
130	2.9	3.8	4.8	11.6	13.7	15.7
150	2.5	3.2	4.2	10.1	11.9	13.7
170	2.2	2.9	3.7	8.9	10.5	12.2
200	1.9	2.4	3.1	7.6	9.0	10.4
250	1.5	1.9	2.5	6.1	7.2	8.4
300	1.2	1.6	2.1	5.1	6.0	7.0
500	.75	.96	1.2	3.1	3.6	4.2
1000	.37	.48	.62	1.5	1.8	2.1

Note.—For mode of use see Examples 1 to 7.   *N* = *total number of individuals in sample.*
P = *probability.*   For *N* > 1000, divide values for *N* = 1000 by *N*/1000.

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 11

N	.005	.025	.10	.10	.025	.005
22	22.9	28.2	34.6	65.4	71.8	77.1
23	21.8	26.8	32.9	63.0	69.4	74.8
24	20.7	25.6	31.5	60.7	67.2	72.6
25	19.8	24.4	30.1	58.6	65.1	70.6
26	18.9	23.4	28.9	56.7	63.1	68.6
27	18.1	22.4	27.7	54.9	61.2	66.7
28	17.4	21.5	26.6	53.1	59.4	64.9
29	16.7	20.7	25.7	51.5	57.7	63.2
30	16.1	19.9	24.8	50.0	56.1	61.6
31	15.4	19.2	23.9	48.6	54.6	60.0
32	14.9	18.6	23.1	47.2	53.2	58.6
33	14.4	18.0	22.4	45.9	51.8	57.1
34	14.0	17.4	21.7	44.7	50.5	55.8
35	13.5	16.9	21.0	43.5	49.3	54.5
36	13.1	16.4	20.4	42.4	48.1	53.3
37	12.7	15.9	19.8	41.4	47.0	52.1
38	12.4	15.4	19.3	40.4	45.9	51.0
39	12.0	15.0	18.8	39.4	44.9	49.9
40	11.7	14.6	18.3	38.5	43.9	48.8
42	11.1	13.9	17.4	36.8	42.0	46.9
45	10.3	12.9	16.2	34.5	39.5	44.2
47	9.8	12.3	15.5	33.2	38.0	42.6
50	9.2	11.5	14.5	31.3	36.0	40.3
52	8.8	11.1	13.9	30.2	34.7	39.0
55	8.3	10.4	13.1	28.6	33.0	37.1
60	7.6	9.5	12.0	26.4	30.4	34.3
65	7.0	8.8	11.1	24.4	28.3	31.9
70	6.5	8.1	10.3	22.8	26.4	29.9
75	6.0	7.6	9.6	21.3	24.7	28.0
80	5.6	7.1	9.0	20.0	23.3	26.4
90	5.0	6.3	7.9	17.9	20.8	23.7
100	4.5	5.6	7.1	16.1	18.8	21.5
110	4.0	5.1	6.5	14.7	17.2	19.6
120	3.7	4.7	5.9	13.5	15.8	18.1
130	3.4	4.3	5.5	12.5	14.6	16.7
150	2.9	3.7	4.7	10.8	12.7	14.6
170	2.6	3.3	4.2	9.6	11.3	12.9
200	2.2	2.8	3.5	8.2	9.6	11.1
250	1.7	2.2	2.8	6.6	7.7	8.9
300	1.5	1.8	2.4	5.5	6.5	7.4
400	1.1	1.4	1.8	4.1	4.9	5.6
500	.87	1.1	1.4	3.3	3.9	4.5
1000	.43	.55	.70	1.7	2.0	2.3

Note.—For mode of use see Examples 1 to 7. N = total number of individuals in sample. P = probability. For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N \ P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 12

N	.005	.025	.10	.10	.025	.005
24	24.0	29.1	35.3	64.7	70.9	76.0
25	22.8	27.8	33.8	62.5	68.7	73.9
26	21.8	26.6	32.4	60.4	66.6	71.9
27	20.9	25.5	31.1	58.5	64.7	70.0
28	20.0	24.5	29.9	56.6	62.8	68.1
29	19.2	23.5	28.8	54.9	61.1	66.4
30	18.5	22.7	27.7	53.3	59.4	64.7
31	17.8	21.9	26.8	51.8	57.8	63.1
32	17.2	21.1	25.9	50.4	56.3	61.6
33	16.6	20.4	25.1	49.0	54.9	60.1
34	16.1	19.8	24.3	47.7	53.5	58.7
35	15.6	19.2	23.5	46.5	52.2	57.3
36	15.1	18.6	22.9	45.3	51.0	56.1
37	14.6	18.0	22.2	44.2	49.8	54.8
38	14.2	17.5	21.6	43.1	48.7	53.7
39	13.8	17.0	21.0	42.1	47.6	52.5
40	13.4	16.6	20.4	41.2	46.5	51.4
42	12.7	15.7	19.4	39.3	44.6	49.4
45	11.8	14.6	18.1	36.9	41.9	46.6
47	11.3	14.0	17.3	35.5	40.4	44.9
50	10.6	13.1	16.2	33.5	38.2	42.6
52	10.1	12.5	15.6	32.3	36.8	41.1
55	9.5	11.8	14.7	30.6	35.0	39.2
60	8.7	10.8	13.4	28.2	32.3	36.3
65	8.0	9.9	12.4	26.1	30.0	33.7
70	7.4	9.2	11.5	24.3	28.0	31.6
75	6.9	8.5	10.7	22.8	26.3	29.6
80	6.4	8.0	10.0	21.4	24.7	27.9
90	5.7	7.1	8.9	19.1	22.1	25.0
100	5.1	6.4	8.0	17.3	20.0	22.7
110	4.6	5.8	7.2	15.7	18.3	20.7
120	4.2	5.3	6.6	14.5	16.8	19.1
130	3.9	4.9	6.1	13.4	15.6	17.7
150	3.4	4.2	5.3	11.6	13.6	15.5
170	3.0	3.7	4.6	10.3	12.0	13.7
200	2.5	3.1	3.9	8.8	10.2	11.7
250	2.0	2.5	3.2	7.0	8.2	9.4
300	1.7	2.1	2.6	5.9	6.9	7.9
400	1.2	1.6	2.0	4.4	5.2	5.9
500	.99	1.2	1.6	3.5	4.2	4.8
1000	.50	.62	.78	1.8	2.1	2.4

Note.—For mode of use see *Examples 1 to 7.* *N* = *total number of individuals in sam* ;
P = probability. For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'S IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 13

N\P	.005	.025	.10	.10	.025	.005
26	24.9	29.9	35.9	64.1	70.1	75.1
27	23.8	28.7	34.5	62.0	68.0	73.2
28	22.8	27.5	33.1	60.1	66.1	71.2
29	21.9	26.4	31.9	58.3	64.3	69.4
30	21.1	25.5	30.8	56.6	62.6	67.7
31	20.3	24.6	29.7	55.0	60.9	66.1
32	19.6	23.7	28.7	53.5	59.3	64.5
33	18.9	.22.9	27.8	52.0	57.8	63.0
34	18.3	22.2	26.9	50.7	56.4	61.5
35	17.7	21.5	26.1	49.4	55.1	60.1
36	17.2	20.8	25.3	48.2	53.8	58.8
37	16.6	20.2	24.6	47.0	52.5	57.5
38	16.2	19.6	23.9	45.9	51.3	56.3
39	15.7	19.1	23.3	44.8	50.2	55.1
40	15.3	18.6	22.6	43.8	49.1	54.0
42	14.5	17.6	21.5	41.8	47.1	51.9
45	13.4	16.4	20.0	39.3	44.3	48.9
47	12.8	15.6	19.1	37.7	42.6	47.2
50	12.0	14.6	17.9	35.6	40.3	44.7
52	11.5	14.0	17.2	34.3	38.9	43.2
55	10.8	13.2	16.2	32.6	37.0	41.2
60	9.9	12.1	14.9	30.0	34.2	38.1
65	9.0	11.1	13.7	27.8	31.8	35.5
70	8.4	10.3	12.7	25.9	29.7	33.2
75	7.8	9.6	11.8	24.2	27.8	31.2
80	7.3	9.0	11.1	22.8	26.2	29.4
85	6.8	8.4	10.4	21.5	24.7	27.8
90	6.4	7.9	9.8	20.4	23.4	26.4
95	6.1	7.5	9.3	19.3	22.3	25.1
100	5.8	7.1	8.8	18.4	21.2	23.9
110	5.2	6.4	8.0	16.8	19.4	21.9
120	4.8	5.9	7.3	15.4	17.8	20.1
130	4.4	5.4	6.7	14.2	16.5	18.7
150	3.8	4.7	5.8	12.3	14.4	16.3
170	3.3	4.1	5.1	11.0	12.7	14.4
200	2.8	3.5	4.4	9.3	10.9	12.4
250	2.3	2.8	3.5	7.5	8.7	9.9
300	1.9	2.3	2.9	6.3	7.3	8.3
400	1.4	1.7	2.2	4.7	5.5	6.3
500	1.1	1.4	1.7	3.8	4.4	5.0
1000	.56	.69	.87	1.9	2.2	2.5

Note.—*For mode of use see Examples 1 to 7. N = total number of individuals in sample.*
= probability. For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 14

N	.005	.025	.10	.10	.025	.005
28	25.7	30.6	36.5	63.5	69.3	74.3
29	24.7	29.5	35.1	61.6	67.5	72.4
30	23.7	28.4	33.8	59.9	65.7	70.7
31	22.9	27.3	32.7	58.2	64.0	69.0
32	22.0	26.4	31.6	56.6	62.3	67.3
33	21.3	25.5	30.5	55.1	60.8	65.8
34	20.6	24.7	29.6	53.6	59.3	64.3
35	19.9	23.9	28.7	52.3	57.9	62.9
36	19.3	23.2	27.8	51.0	56.5	61.5
37	18.7	22.5	27.0	49.7	55.2	60.2
38	18.1	21.8	26.3	48.5	54.0	58.9
39	17.6	21.2	25.5	47.4	52.8	57.7
40	17.1	20.6	24.9	46.3	51.7	56.5
42	16.2	19.6	23.6	44.3	49.5	54.3
45	15.0	18.2	22.0	41.6	46.6	51.3
47	14.4	17.3	21.0	40.0	44.9	49.4
50	13.4	16.2	19.7	37.7	42.5	46.9
52	12.9	15.6	18.9	36.4	41.0	45.3
55	12.1	14.7	17.8	34.5	39.0	43.2
57	11.7	14.1	17.2	33.4	37.7	41.8
60	11.0	13.4	16.3	31.8	36.0	40.0
65	10.1	12.3	15.0	29.5	33.5	37.2
70	9.4	11.4	13.9	27.5	31.3	34.9
75	8.7	10.6	12.9	25.7	29.3	32.7
80	8.1	9.9	12.1	24.2	27.6	30.9
85	7.6	9.3	11.4	22.8	26.1	29.2
90	7.2	8.8	10.7	21.6	24.7	27.7
100	6.5	7.9	9.6	19.5	22.4	25.1
110	5.9	7.1	8.8	17.8	20.4	23.0
120	5.3	6.5	8.0	16.3	18.8	21.2
130	4.9	6.0	7.4	15.1	17.4	19.6
140	4.6	5.6	6.8	14.1	16.2	18.3
150	4.3	5.2	6.4	13.1	15.2	17.1
170	3.7	4.6	5.6	11.6	13.4	15.2
200	3.2	3.9	4.8	9.9	11.5	13.0
250	2.5	3.1	3.8	8.0	9.2	10.5
300	2.1	2.6	3.2	6.6	7.7	8.7
400	1.6	1.9	2.4	5.0	5.8	6.6
500	1.3	1.5	1.9	4.0	4.7	5.3
1000	.63	.77	.95	2.0	2.3	2.7

Note.—For mode of use see Examples 1 to 7. N = total number of individuals in sample.
P = probability. For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF A's IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 15

30	26.5	31.3	37.0	63.0	68.7	73.5
31	25.5	30.2	35.7	61.3	66.9	71.8
32	24.6	29.1	34.5	59.6	65.3	70.1
33	23.7	28.2	33.3	58.0	63.7	68.5
34	22.9	27.3	32.3	56.5	62.1	67.0
35	22.1	26.4	31.3	55.1	60.7	65.5
36	21.5	25.6	30.4	53.7	59.2	64.1
37	20.8	24.9	29.5	52.4	57.9	62.8
38	20.2	24.2	28.6	51.2	56.6	61.4
39	19.6	23.5	27.9	50.0	55.4	60.2
40	19.1	22.8	27.1	48.9	54.2	59.0
42	18.0	21.7	25.8	46.8	52.0	56.7
45	16.7	20.1	24.0	43.9	49.0	53.6
47	15.9	19.2	22.9	42.2	47.1	51.6
50	14.9	18.0	21.5	39.8	44.6	49.0
52	14.3	17.2	20.6	38.4	43.1	47.4
55	13.4	16.2	19.4	36.4	41.0	45.2
57	12.9	15.6	18.7	35.2	39.7	43.8
60	12.2	14.8	17.7	33.6	37.9	41.8
65	11.2	13.6	16.3	31.1	35.2	39.0
70	10.4	12.6	15.1	29.0	32.9	36.5
75	9.7	11.7	14.1	27.2	30.8	34.3
80	9.0	11.0	13.2	25.5	29.0	32.3
85	8.5	10.3	12.4	24.1	27.4	30.6
90	8.0	9.7	11.7	22.8	26.0	29.0
100	7.2	8.7	10.5	20.6	23.5	26.3
110	6.5	7.9	9.5	18.8	21.5	24.1
120	5.9	7.2	8.7	17.3	19.8	22.2
130	5.5	6.6	8.0	16.0	18.3	20.6
140	5.1	6.2	7.5	14.9	17.1	19.2
150	4.7	5.7	7.0	13.9	16.0	18.0
170	4.1	5.0	6.1	12.3	14.1	15.9
200	3.5	4.3	5.2	10.5	12.1	13.6
250	2.8	3.4	4.2	8.4	9.7	11.0
300	2.3	2.8	3.5	7.0	8.1	9.2
400	1.7	2.1	2.6	5.3	6.1	6.9
500	1.4	1.7	2.1	4.2	4.9	5.6
1000	.69	.84	1.0	2.1	2.5	2.8

*Note.—For mode of use see Examples 1 to 7. N = total number of individuals in sample.
P = probability. For N > 1000, divide values for N = 1000 by N/1000.*

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 16

N\P	.005	.025	.10	.10	.025	.005
32	27.2	31.9	37.4	62.6	68.1	72.8
33	26.3	30.8	36.2	60.9	66.4	71.1
34	25.4	29.8	35.0	59.4	64.8	69.6
35	24.5	28.9	34.0	57.9	63.3	68.1
36	23.7	28.0	32.9	56.4	61.9	66.6
37	23.0	27.2	32.0	55.1	60.5	55.2
38	22.3	26.4	31.1	53.8	59.2	63.9
39	21.7	25.7	30.2	52.6	57.9	62.6
40	21.1	25.0	29.4	51.4	56.7	61.3
42	19.9	23.7	27.9	49.2	54.4	59.0
45	18.5	22.0	26.0	46.2	51.2	55.8
47	17.6	21.0	24.8	44.4	49.3	53.8
50	16.4	19.6	23.2	41.9	46.7	51.1
52	15.8	18.8	22.3	40.4	45.1	49.4
55	14.8	17.7	21.0	38.4	42.9	47.1
57	14.3	17.1	20.3	37.1	41.5	45.6
60	13.5	16.2	19.2	35.4	39.6	43.6
62	13.0	15.6	18.6	34.3	38.5	42.4
65	12.4	14.9	17.7	32.8	36.9	40.7
67	12.0	14.4	17.1	31.9	35.9	39.6
70	11.4	13.8	16.4	30.6	34.4	38.1
75	10.6	12.8	15.3	28.6	32.3	35.8
80	9.9	12.0	14.3	26.9	30.4	33.8
85	9.3	11.2	13.4	25.4	28.8	31.9
90	8.8	10.6	12.7	24.0	27.3	30.3
100	7.9	9.5	11.4	21.7	24.7	27.5
110	7.1	8.6	10.3	19.8	22.5	25.2
120	6.5	7.9	9.4	18.2	20.7	23.2
130	6.0	7.2	8.7	16.8	19.2	21.5
140	5.6	6.7	8.1	15.7	17.9	20.0
150	5.2	6.2	7.5	14.6	16.7	18.8
170	4.6	5.5	6.6	13.0	14.8	16.7
200	3.9	4.7	5.6	11.0	12.7	14.2
250	3.1	3.7	4.5	8.9	10.2	11.5
300	2.6	3.1	3.7	7.4	8.5	9.6
400	1.9	2.3	2.8	5.6	6.4	7.2
500	1.5	1.8	2.2	4.5	5.1	5.8
1000	.76	.92	1.1	2.2	2.6	2.9

Note.—*For mode of use see Examples 1 to 7.   N = total number of individuals in sample.* = *probability.   For N > 1000, divide values for N = 1000 by N/1000.*

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 17

N\P	.005	.025	.10	.10	.025	.005
34	27.9	32.5	37.8	62.2	67.5	72.1
35	27.0	31.4	36.7	60.6	66.0	70.6
36	26.1	30.5	35.6	59.1	64.5	69.1
37	25.3	29.6	34.5	57.7	63.0	67.7
38	24.5	28.7	33.5	56.4	61.7	66.3
39	23.8	27.9	32.6	55.1	60.3	65.0
40	23.1	27.2	31.7	53.9	59.1	63.7
41	22.5	26.4	30.9	52.7	57.9	62.4
42	21.9	25.8	30.1	51.5	56.7	61.3
43	21.3	25.1	29.4	50.5	55.6	60.1
44	20.8	24.5	28.7	49.4	54.5	59.0
45	20.3	23.9	28.0	48.4	53.4	57.9
46	19.8	23.3	27.4	47.5	52.4	56.9
47	19.3	22.8	26.7	46.5	51.5	55.9
48	18.9	22.3	26.2	45.6	50.5	54.9
49	18.4	21.8	25.6	44.8	49.6	54.0
50	18.0	21.3	25.1	44.0	48.7	53.1
52	17.3	20.5	24.1	42.4	47.1	51.4
55	16.2	19.3	22.7	40.2	44.8	49.0
57	15.6	18.6	21.9	38.9	43.4	47.5
60	14.8	17.6	20.7	37.1	41.4	45.4
62	14.3	17.0	20.0	36.0	40.2	44.1
65	13.6	16.1	19.1	34.4	38.5	42.3
67	13.1	15.6	18.5	33.4	37.5	41.2
70	12.5	14.9	17.6	32.1	36.0	39.6
75	11.6	13.9	16.4	30.1	33.8	37.3
80	10.9	13.0	15.4	28.3	31.8	35.2
85	10.2	12.2	14.5	26.7	30.1	33.3
90	9.6	11.5	13.6	25.2	28.5	31.6
100	8.6	10.3	12.2	22.8	25.8	28.7
110	7.8	9.3	11.1	20.8	23.6	26.2
120	7.1	8.5	10.2	19.1	21.7	24.2
130	6.6	7.8	9.4	17.7	20.1	22.4
140	6.1	7.3	8.7	16.5	18.7	20.9
150	5.6	6.8	8.1	15.4	17.5	19.6
170	5.0	6.0	7.1	13.6	15.5	17.4
200	4.2	5.0	6.1	11.6	13.3	14.9
250	3.3	4.0	4.8	9.3	10.7	12.0
300	2.8	3.3	4.0	7.8	8.9	10.0
400	2.1	2.5	3.0	5.9	6.7	7.6
500	1.7	2.0	2.4	4.7	5.4	6.1
1000	.83	.99	1.2	2.4	2.7	3.1

Note.—For mode of use see Examples 1 to 7. N = total number of individuals in sample.
= probability. For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Number of A's in sample = 18

N\P	.005	.025	.10	.10	.025	.005
36	28.5	33.0	38.2	61.8	67.0	71.5
37	27.6	32.0	37.1	60.3	65.5	70.0
38	26.8	31.1	36.0	58.9	64.1	68.6
39	26.0	30.2	35.0	57.6	62.8	67.3
40	25.3	29.4	34.1	56.3	61.5	66.0
41	24.5	28.6	33.2	55.1	60.2	64.7
42	23.9	27.8	32.4	53.9	59.0	63.5
43	23.3	27.1	31.6	52.8	57.8	62.3
44	22.7	26.5	30.8	51.7	56.7	61.2
45	22.1	25.8	30.1	50.6	55.6	60.1
46	21.6	25.2	29.4	49.6	54.6	59.0
47	21.0	24.6	28.7	48.7	53.6	58.0
48	20.6	24.1	28.1	47.8	52.6	57.0
49	20.1	23.6	27.5	46.9	51.7	56.0
50	19.6	23.1	26.9	46.0	50.8	55.1
52	18.8	22·1	25.8	44.4	49.1	53.3
55	17.7	20.8	24.3	42.1	46.7	50.8
57	17.0	20.0	23.4	40.8	45.2	49.3
60	16.1	19.0	22.2	38.8	43.2	47.2
62	15.5	18.3	21.5	37.7	41.9	45.8
65	14.8	17.4	20.4	36.0	40.2	44.0
67	14.3	16.9	19.8	35.0	39.1	42.8
70	13.6	16.1	18.9	33.6	37.5	41.2
75	12.7	15.0	17.6	31.5	35.2	38.7
80	11.8	14.0	16.5	29.6	33.2	36.6
85	11.1	13.1	15.5	27.9	31.4	34.6
90	10.4	12.4	14.6	26.4	29.7	32.8
100	9.3	11.1	13.1	23.9	26.9	29.8
110	8.5	10.0	11.9	21.8	24.6	27.3
120	7.7	9.2	10.9	20.0	22.7	25.2
130	7.1	8.5	10.0	18.5	21.0	23.3
140	6.6	7.8	9.3	17.3	19.6	21.8
150	6.1	7.3	8.7	16.1	18.3	20.4
170	5.4	6.4	7.6	14.3	16.2	18.1
200	4.6	5.4	6.5	12.2	13.9	15.5
250	3.6	4.3	5.2	9.8	11.1	12.5
300	3.0	3.6	4.3	8.2	9.3	10.4
400	2.3	2.7	3.2	6.1	7.0	7.9
500	1.8	2.2	2.6	4.9	5.6	6.3
1000	.90	1.1	1.3	2.5	2.8	3.2

*Note.—For mode of use see Examples 1 to 7. N = total number of individuals in sample.
P = probability. For $N > 1000$, divide values for $N = 1000$ by $N/1000$.*

TABLE IB—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Continued*

	Lower limits (%)			Upper limits (%)		
N＼P	.005	.025	.10	.10	.025	.005

Number of A's in sample = 19

38	29.1	33.5	38.6	61.4	66.5	70.9
39	28.2	32.5	37.5	60.1	65.1	69.5
40	27.4	31.6	36.5	58.7	63.8	68.2
41	26.6	30.8	35.5	57.5	62.5	66.9
42	25.9	30.0	34.6	56.2	61.3	65.7
43	25.2	29.2	33.7	55.1	60.1	64.5
44	24.6	28.5	32.9	53.9	58.9	63.3
45	24.0	27.8	32.1	52.9	57.8	62.2
46	23.4	27.1	31.4	51.8	56.7	61.1
47	22.8	26.5	30.7	50.8	55.7	60.0
48	22.3	25.9	30.0	49.9	54.7	59.0
49	21.8	25.3	29.4	48.9	53.7	58.0
50	21.3	24.8	28.7	48.0	52.8	57.0
52	20.4	23.8	27.6	46.3	51.0	55.2
55	19.2	22.4	26.0	44.0	48.5	52.7
57	18.4	21.5	25.0	42.6	47.0	51.1
60	17.4	20.4	23.7	40.6	44.9	48.9
62	16.8	19.7	22.9	39.4	43.6	47.5
65	16.0	18.7	21.8	37.6	41.8	45.6
67	15.5	18.1	21.2	36.6	40.7	44.4
70	14.7	17.3	20.2	35.1	39.1	42.7
75	13.7	16.1	18.8	32.9	36.7	40.2
80	12.8	15.0	17.6	30.9	34.6	37.9
85	12.0	14.1	16.5	29.2	32.7	35.9
90	11.3	13.3	15.6	27.6	31.0	34.1
100	10.1	11.9	14.0	25.0	28.1	31.0
110	9.1	10.8	12.7	22.8	25.6	28.4
120	8.3	9.9	11.6	21.0	23.6	26.1
130	7.7	9.1	10.7	19.4	21.9	24.2
140	7.1	8.4	9.9	18.0	20.4	22.6
150	6.6	7.8	9.3	16.9	19.1	21.2
170	5.8	6.9	8.2	14.9	16.9	18.8
200	4.9	5.8	6.9	12.7	14.4	16.1
250	3.9	4.7	5.5	10.2	11.6	13.0
300	3.3	3.9	4.6	8.5	9.7	10.9
400	2.4	2.9	3.4	6.4	7.3	8.2
500	1.9	2.3	2.7	5.1	5.9	6.6
1000	.97	1.1	1.4	2.6	3.0	3.3

Note.—For mode of use see Examples 1 to 7.   N = total number of individuals in sample.
= probability.   For N > 1000, divide values for N = 1000 by N/1000.

TABLE IB—*Concluded*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 1 TO 20—*Concluded*

	Lower limits (%)			Upper limits (%)		
N\P	.005	.025	.10	.10	.025	.005

Number of A's in sample = 20

40	29.6	33.9	38.9	61.1	66.1	70.4
41	28.8	33.0	37.8	59.8	64.8	69.1
42	28.0	32.1	36.9	58.6	63.5	67.8
43	27.3	31.3	35.9	57.3	62.3	66.6
44	26.5	30.5	35.1	56.2	61.1	65.4
45	25.9	29.8	34.2	55.0	59.9	64.2
46	25.2	29.1	33.4	54.0	58.8	63.1
47	24.6	28.4	32.7	52.9	57.8	62.0
48	24.0	27.8	32.0	51.9	56.7	61.0
49	23.5	27.1	31.3	51.0	55.7	60.0
50	23.0	26.6	30.6	50.0	54.8	59.0
51	22.5	26.0	30.0	49.1	53.8	58.0
52	22.0	25.5	29.4	48.3	52.9	57.1
53	21.5	24.9	28.8	47.4	52.1	56.2
54	21.1	24.4	28.2	46.6	51.2	55.3
55	20.7	24.0	27.7	45.8	50.4	54.5
56	20.3	23.5	27.2	45.1	49.6	53.7
57	19.9	23.1	26.6	44.4	48.8	52.9
58	19.5	22.6	26.2	43.7	48.1	52.1
59	19.1	22.2	25.7	43.0	47.3	51.3
60	18.8	21.8	25.3	42.3	46.6	50.6
62	18.1	21.1	24.4	41.0	45.3	49.2
65	17.2	20.0	23.2	39.3	43.4	47.2
67	16.7	19.4	22.5	38.2	42.2	46.0
70	15.9	18.5	21.5	36.6	40.6	44.3
75	14.7	17.2	20.0	34.3	38.1	41.6
80	13.8	16.1	18.7	32.3	35.9	39.3
85	12.9	15.1	17.6	30.5	34.0	37.2
90	12.1	14.2	16.6	28.8	32.2	35.3
100	10.9	12.7	14.9	26.1	29.2	32.1
110	9.8	11.5	13.5	23.8	26.7	29.4
120	9.0	10.5	12.4	21.9	24.6	27.1
130	8.3	9.7	11.4	20.2	22.8	25.2
140	7.6	9.0	10.6	18.8	21.2	23.5
150	7.1	8.4	9.8	17.6	19.8	22.0
170	6.3	7.4	8.7	15.6	17.6	19.5
200	5.3	6.2	7.4	13.3	15.0	16.7
250	4.2	5.0	5.9	10.7	12.1	13.5
300	3.5	4.1	4.9	8.9	10.1	11.3
400	2.6	3.1	3.7	6.7	7.6	8.5
500	2.1	2.5	2.9	5.4	6.1	6.8
1000	1.0	1.2	1.5	2.7	3.1	3.4

Note.—For mode of use see Examples 1 to 7. N = total number of individuals in sample.
P = probability. For N > 1000, divide values for N = 1000 by N/1000.

TABLE II

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF A'S IN SAMPLE: 20 AND OVER

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Percentage of A's in sample = 50

N\P	.005	.025	.10	.10	.025	.005
40	29.6	33.9	38.9	61.1	66.1	70.4
43	30.4	34.5	39.3	60.7	65.5	69.6
47	31.2	35.2	39.8	60.2	64.8	68.8
50	31.8	35.7	40.1	59.9	64.3	68.2
55	32.6	36.3	40.6	59.4	63.7	67.4
60	33.4	36.9	41.1	58.9	63.1	66.6
65	34.0	37.5	41.4	58.6	62.5	66.0
70	34.6	37.9	41.8	58.2	62.1	65.4
75	35.1	38.4	42.1	57.9	61.6	64.9
80	35.6	38.7	42.3	57.7	61.3	64.4
85	36.0	39.1	42.6	57.4	60.9	64.0
90	36.4	39.4	42.8	57.2	60.6	63.6
100	37.1	40.0	43.2	56.8	60.0	62.9
110	37.7	40.4	43.5	56.5	59.6	62.3
120	38.2	40.9	43.8	56.2	59.1	61.8
130	38.7	41.2	44.1	55.9	58.8	61.3
150	39.5	41.8	44.5	55.5	58.2	60.5
170	40.1	42.3	44.9	55.1	57.7	59.9
200	40.9	43.0	45.3	54.7	57.0	59.1
220	41.3	43.3	45.5	54.5	56.7	58.7
250	41.9	43.7	45.8	54.2	56.3	58.1
300	42.6	44.3	46.2	53.8	55.7	57.4
350	43.1	44.7	46.5	53.5	55.3	56.9
400	43.6	45.0	46.7	53.3	55.0	56.4
500	44.2	45.6	47.1	52.9	54.4	55.8
600	44.7	46.0	47.3	52.7	54.0	55.3
700	45.1	46.3	47.5	52.5	53.7	54.9
1 M	45.9	46.9	47.9	52.1	53.1	54.1
1.5 M	46.7	47.5	48.3	51.7	52.5	53.3
2 M	47.1	47.8	48.5	51.5	52.2	52.9
3 M	47.6	48.2	48.8	51.2	51.8	52.4
5 M	48.2	48.6	49.1	50.9	51.4	51.8
10 M	48.7	49.0	49.4	50.6	51.0	51.3
20 M	49.1	49.3	49.5	50.5	50.7	50.9
50 M	49.4	49.6	49.7	50.3	50.4	50.6
100 M	49.6	49.7	49.8	50.2	50.3	50.4

Note.—For mode of use see Examples 8 to 12. M = 1000. N = total number of individuals in the sample. P = probability.

TABLE II—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF A's IN SAMPLE: 20 AND OVER—*Continued*

	Lower limits (%)			Upper limits (%)		
N\P	.005	.025	.10	.10	.025	.005

Percentage of A's in sample = 45

40	25.3	29.4	34.1	56.3	61.5	66.0
43	25.9	29.9	34.5	55.9	60.8	65.2
47	26.7	30.6	35.0	55.3	60.1	64.3
50	27.3	31.0	35.3	55.0	59.6	63.7
55	28.1	31.7	35.8	54.5	58.9	62.8
60	28.8	32.3	36.2	54.0	58.3	62.0
65	29.4	32.8	36.6	53.7	57.7	61.3
70	30.0	33.2	36.9	53.3	57.2	60.7
75	30.5	33.6	37.2	53.0	56.8	60.2
80	30.9	34.0	37.5	52.7	56.4	59.7
85	31.3	34.3	37.7	52.5	56.1	59.2
90	31.7	34.6	37.9	52.3	55.7	58.8
100	32.4	35.2	38.3	51.9	55.2	58.1
110	33.0	35.6	38.6	51.5	54.7	57.5
120	33.5	36.0	38.9	51.2	54.2	56.9
130	33.9	36.4	39.2	51.0	53.9	56.5
150	34.7	37.0	39.6	50.5	53.2	55.6
170	35.3	37.5	39.9	50.2	52.7	55.0
200	36.0	38.1	40.3	49.8	52.1	54.2
220	36.5	38.4	40.6	49.5	51.8	53.8
250	37.0	38.8	40.8	49.2	51.3	53.2
300	37.7	39.3	41.2	48.8	50.8	52.5
350	38.2	39.8	41.5	48.5	50.3	51.9
400	38.6	40.1	41.7	48.3	50.0	51.5
500	39.3	40.6	42.1	47.9	49.4	50.8
700	40.2	41.3	42.5	47.5	48.7	49.9
1 M	41.0	41.9	43.0	47.1	48.1	49.1
1.5 M	41.7	42.5	43.3	46.7	47.5	48.3
2 M	42.1	42.8	43.6	46.5	47.2	47.9
3 M	42.7	43.2	43.8	46.2	46.8	47.3
5 M	43.2	43.6	44.1	45.9	46.4	46.8
10 M	43.7	44.0	44.4	45.6	46.0	46.3
20 M	44.1	44.3	44.5	45.5	45.7	45.9
50 M	44.4	44.6	44.7	45.3	45.4	45.6
100 M	44.6	44.7	44.8	45.2	45.3	45.4

Note.—For mode of use see Examples 8 to 12. $M = 1000$. $N = $ total number of individuals in the sample. $P = $ probability.

TABLE II—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 20 AND OVER—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Percentage of A's in sample = 40

N\P	.005	.025	.10	.10	.025	.005
43	21.7	25.5	29.8	50.9	56.0	60.5
47	22.5	26.1	30.3	50.4	55.3	59.6
50	23.0	26.6	30.6	50.0	54.8	59.0
55	23.7	27.2	31.1	49.5	54.0	58.0
60	24.4	27.7	31.5	49.1	53.4	57.2
65	25.0	28.2	31.8	48.7	52.8	56.5
70	25.5	28.6	32.1	48.3	52.3	55.9
75	26.0	29.0	32.4	48.0	51.9	55.3
80	26.4	29.4	32.7	47.7	51.5	54.8
85	26.8	29.7	32.9	47.5	51.1	54.3
90	27.2	30.0	33.1	47.3	50.8	53.9
100	27.8	30.5	33.5	46.9	50.2	53.2
110	28.3	30.9	33.8	46.5	49.7	52.5
120	28.8	31.3	34.1	46.2	49.3	52.0
130	29.2	31.6	34.3	46.0	48.9	51.5
140	29.6	31.9	34.5	45.7	48.5	51.1
150	30.0	32.2	34.7	45.5	48.2	50.7
170	30.6	32.7	35.0	45.2	47.7	50.0
200	31.3	33.2	35.4	44.7	47.1	49.2
250	32.2	34.0	35.9	44.2	46.3	48.2
300	32.9	34.5	36.3	43.8	45.7	47.5
350	33.4	34.9	36.6	43.5	45.3	46.9
400	33.8	35.2	36.8	43.3	44.9	46.4
500	34.4	35.7	37.1	42.9	44.4	45.8
700	35.3	36.4	37.6	42.5	43.7	44.8
1 M	36.1	37.0	38.0	42.0	43.1	44.0
1.5 M	36.8	37.5	38.4	41.7	42.5	43.3
2 M	37.2	37.9	38.6	41.4	42.2	42.8
3 M	37.7	38.2	38.8	41.2	41.8	42.3
5 M	38.2	38.6	39.1	40.9	41.4	41.8
10 M	38.7	39.0	39.4	40.6	41.0	41.3
20 M	39.1	39.3	39.6	40.4	40.7	40.9
50 M	39.4	39.6	39.7	40.3	40.4	40.6
100 M	39.6	39.7	39.8	40.2	40.3	40.4

Note.—For mode of use see Examples 8 to 12. M = 1000. N = total number of individuals in the sample. P = probability.

TABLE II—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 20 AND OVER—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Percentage of A's in sample = 35

N\P	.005	.025	.10	.10	.025	.005
50	18.8	22.2	26.0	45.0	49.8	54.1
55	19.5	22.8	26.4	44.5	49.0	53.1
60	20.1	23.3	26.8	44.0	48.4	52.3
65	20.7	23.7	27.1	43.6	47.8	51.5
70	21.2	24.1	27.4	43.3	47.3	50.9
75	21.6	24.5	27.7	43.0	46.8	50.3
80	22.0	24.8	27.9	42.7	46.4	49.8
90	22.7	25.4	28.3	42.2	45.7	48.9
100	23.3	25.9	28.7	41.8	45.1	48.1
110	23.8	26.3	29.0	41.4	44.6	47.5
120	24.3	26.6	29.3	41.1	44.2	46.9
130	24.7	27.0	29.5	40.9	43.8	46.4
140	25.0	27.2	29.7	40.6	43.5	46.0
150	25.4	27.5	29.9	40.4	43.1	45.6
170	25.9	28.0	30.2	40.1	42.6	44.9
200	26.6	28.5	30.6	39.7	42.0	44.1
250	27.5	29.2	31.1	39.1	41.2	43.1
300	28.1	29.7	31.4	38.8	40.7	42.4
350	28.6	30.1	31.7	38.5	40.2	41.8
400	29.0	30.4	31.9	38.2	39.9	41.4
500	29.6	30.9	32.2	37.9	39.3	40.7
700	30.4	31.5	32.7	37.4	38.6	39.8
1 M	31.2	32.1	33.0	37.0	38.0	39.0
1.5 M	31.9	32.6	33.4	36.6	37.5	38.2
2 M	32.3	32.9	33.6	36.4	37.1	37.8
3 M	32.8	33.3	33.9	36.1	36.7	37.3
5 M	33.3	33.7	34.1	35.9	36.3	36.8
10 M	33.8	34.1	34.4	35.6	35.9	36.2
20 M	34.1	34.3	34.6	35.4	35.7	35.9
50 M	34.5	34.6	34.7	35.3	35.4	35.6
100 M	34.6	34.7	34.8	35.2	35.3	35.4

Note.—*For mode of use see Examples 8 to 12.   M = 1000.   N = total number of individuals in the sample.   P = probability.*

TABLE II—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Percentage of A's in sample = 30

N\P	.005	.025	.10	.10	.025	.005
55	15.5	18.5	21.9	39.3	43.8	48.0
60	16.1	19.0	22.2	38.8	43.2	47.2
65	16.6	19.4	22.5	38.5	42.6	46.4
70	17.0	19.8	22.8	38.1	42.1	45.8
75	17.4	20.1	23.1	37.8	41.6	45.2
80	17.8	20.4	23.3	37.5	41.2	44.6
85	18.1	20.7	23.5	37.3	40.9	44.2
90	18.4	20.9	23.7	37.0	40.5	43.7
95	18.7	21.1	23.8	36.8	40.2	43.3
100	19.0	21.4	24.0	36.6	39.9	43.0
110	19.5	21.7	24.3	36.3	39.4	42.3
120	19.9	22.1	24.5	36.0	39.0	41.7
130	20.3	22.4	24.8	35.7	38.6	41.2
150	20.9	22.9	25.1	35.3	38.0	40.4
170	21.4	23.3	25.4	35.0	37.4	39.7
200	22.1	23.8	25.8	34.5	36.8	38.9
250	22.9	24.5	26.2	34.0	36.1	37.9
300	23.5	24.9	26.6	33.6	35.5	37.2
350	23.9	25.3	26.8	33.4	35.1	36.6
400	24.3	25.6	27.0	33.1	34.7	36.2
500	24.9	26.1	27.3	32.8	34.2	35.5
700	25.7	26.7	27.8	32.3	33.5	34.6
1 M	26.3	27.2	28.1	31.9	32.9	33.8
1.5 M	27.0	27.7	28.5	31.6	32.4	33.1
2 M	27.4	28.0	28.7	31.4	32.1	32.7
3 M	27.9	28.4	28.9	31.1	31.7	32.2
5 M	28.3	28.7	29.2	30.8	31.3	31.7
10 M	28.8	29.1	29.4	30.6	30.9	31.2
20 M	29.2	29.4	29.6	30.4	30.6	30.8
50 M	29.5	29.6	29.7	30.3	30.4	30.5
100 M	29.6	29.7	29.8	30.2	30.3	30.4

Percentage of A's in sample = 25

N\P	.005	.025	.10	.10	.025	.005
65	12.7	15.2	18.0	33.2	37.3	41.1
70	13.1	15.5	18.3	32.8	36.8	40.4
75	13.4	15.8	18.5	32.5	36.3	39.8
80	13.8	16.1	18.7	32.3	35.9	39.3
85	14.1	16.3	18.9	32.0	35.5	38.8
90	14.3	16.6	19.1	31.8	35.2	38.4
100	14.8	17.0	19.4	31.4	34.6	37.6
110	15.3	17.3	19.7	31.1	34.1	37.0
120	15.6	17.6	19.9	30.8	33.7	36.4
130	16.0	17.9	20.1	30.5	33.3	35.9

Note.—For mode of use see Examples 8 to 12. M = 1000. N = total number of individuals in the sample. P = probability.

TABLE II—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 20 AND OVER—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Percentage of A's in sample = 25

N\P	.005	.025	.10	.10	.025	.005
150	16.5	18.4	20.4	30.1	32.7	35.1
170	17.0	18.8	20.7	29.8	32.2	34.4
200	17.6	19.2	21.0	29.3	31.6	33.6
250	18.3	19.8	21.5	28.8	30.8	32.6
300	18.9	20.3	21.8	28.5	30.3	31.9
350	19.3	20.6	22.0	28.2	29.9	31.4
400	19.7	20.9	22.2	28.0	29.5	30.9
500	20.2	21.3	22.5	27.7	29.0	30.3
700	20.9	21.9	22.9	27.2	28.4	29.4
1 M	21.6	22.4	23.2	26.8	27.8	28.7
1.5 M	22.2	22.8	23.6	26.5	27.3	28.0
2 M	22.6	23.1	23.8	26.3	27.0	27.6
3 M	23.0	23.5	24.0	26.0	26.6	27.1
5 M	23.4	23.8	24.2	25.8	26.2	26.6
10 M	23.9	24.2	24.4	25.6	25.9	26.1
20 M	24.2	24.4	24.6	25.4	25.6	25.8
50 M	24.5	24.6	24.8	25.2	25.4	25.5
100 M	24.6	24.7	24.8	25.2	25.3	25.4

Percentage of A's in sample = 20

N\P	.005	.025	.10	.10	.025	.005
80	9.9	12.0	14.3	26.9	30.4	33.8
85	10.2	12.2	14.5	26.7	30.1	33.3
90	10.4	12.4	14.6	26.4	29.7	32.8
100	10.9	12.7	14.9	26.1	29.2	32.1
110	11.2	13.1	15.1	25.7	28.7	31.5
120	11.6	13.3	15.3	25.5	28.3	30.9
130	11.9	13.6	15.5	25.2	27.9	30.4
150	12.4	14.0	15.8	24.8	27.3	29.6
170	12.8	14.3	16.1	24.5	26.8	29.0
200·	13.3	14.7	16.4	24.1	26.2	28.2
250	14.0	15.3	16.8	23.6	25.5	27.2
300	14.4	15.7	17.0	23.3	25.0	26.5
350	14.8	16.0	17.3	23.0	24.6	26.0
400	15.1	16.2	17.4	22.8	24.3	25.6
500	15.6	16.6	17.7	22.5	23.8	25.0
700	16.3	17.1	18.1	22.1	23.2	24.2
1 M	16.9	17.6	18.4	21.7	22.6	23.4
1.5 M	17.4	18.0	18.7	21.4	22.1	22.8
2 M	17.8	18.3	18.9	21.2	21.8	22.4
3 M	18.2	18.6	19.1	21.0	21.5	21.9
5 M	18.6	18.9	19.3	20.7	21.1	21.5
10 M	19.0	19.2	19.5	20.5	20.8	21.0
20 M	19.3	19.4	19.6	20.4	20.6	20.7
50 M	19.5	19.7	19.8	20.2	20.4	20.5
100 M	19.7	19.8	19.8	20.2	20.2	20.3

Note.—For mode of use see Examples 8 to 12. M = 1000. N = total number of individuals in the sample. P = probability.

TABLE II—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 20 AND OVER—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Percentage of A's in sample = 15

N	.005	.025	.10	.10	.025	.005
100	7.2	8.7	10.5	20.6	23.5	26.3
110	7.5	9.0	10.7	20.3	23.1	25.7
120	7.7	9.2	10.9	20.0	22.7	25.2
130	8.0	9.4	11.0	19.8	22.3	24.7
150	8.4	9.7	11.3	19.4	21.7	23.9
170	8.7	10.0	11.5	19.1	21.3	23.3
200	9.2	10.4	11.8	18.8	20.7	22.6
250	9.7	10.8	12.1	18.3	20.0	21.7
300	10.1	11.2	12.4	18.0	19.6	21.0
350	10.5	11.5	12.6	17.7	19.2	20.5
400	10.7	11.7	12.7	17.5	18.9	20.1
500	11.2	12.0	13.0	17.3	18.4	19.5
700	11.7	12.5	13.3	16.9	17.9	18.8
1 M	12.2	12.9	13.6	16.6	17.4	18.1
1.5 M	12.7	13.2	13.8	16.3	16.9	17.5
2 M	13.0	13.5	14.0	16.1	16.6	17.2
3 M	13.4	13.7	14.2	15.9	16.3	16.8
5 M	13.7	14.0	14.4	15.7	16.0	16.3
10 M	14.1	14.3	14.5	15.5	15.7	15.9
20 M	14.4	14.5	14.7	15.3	15.5	15.7
50 M	14.6	14.7	14.8	15.2	15.3	15.4
100 M	14.7	14.8	14.9	15.1	15.2	15.3

Percentage of A's in sample = 10

N	.005	.025	.10	.10	.025	.005
130	4.4	5.4	6.7	14.2	16.5	18.7
150	4.7	5.7	7.0	13.9	16.0	18.0
170	5.0	6.0	7.1	13.6	15.5	17.4
200	5.3	6.2	7.4	13.3	15.0	16.7
250	5.7	6.6	7.6	12.9	14.4	15.9
300	6.0	6.9	7.8	12.6	14.0	15.3
350	6.3	7.1	8.0	12.4	13.6	14.8
400	6.5	7.3	8.1	12.2	13.4	14.5
500	6.9	7.5	8.3	11.9	13.0	13.9
700	7.3	7.9	8.6	11.6	12.5	13.3
1 M	7.7	8.2	8.8	11.3	12.0	12.7
1.5 M	8.1	8.5	9.0	11.1	11.6	12.2
2 M	8.3	8.7	9.1	10.9	11.4	11.9
3 M	8.6	9.0	9.3	10.7	11.1	11.5
5 M	8.9	9.2	9.5	10.6	10.9	11.1
10 M	9.2	9.4	9.6	10.4	10.6	10.8
20 M	9.5	9.6	9.7	10.3	10.4	10.6
50 M	9.7	9.7	9.8	10.2	10.3	10.4
100 M	9.8	9.8	9.9	10.1	10.2	10.2

Note.—For mode of use see Examples 8 to 12. M = 1000. N = total number of individuals in the sample. P = probability.

. TABLE II—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 20 AND OVER—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025 ·	.10	.10	.025	.005

Percentage of A's in sample = 7.5

N	.005	.025	.10	.10	.025	.005
200	3.5	4.3	5.2	10.5	12.1	13.6
250	3.9	4.6	5.4	10.1	11.5	12.9
300	4.1	4.8	5.6	9.8	11.1	12.3
350	4.3	5.0	5.7	9.6	10.8	11.9
400	4.5	5.1	5.9	9.5	10.5	11.6
500	4.8	5.4	6.0	9.2	10.2	11.1
700	5.2	5.7	6.2	8.9	9.7	10.4
1 M	5.5	6.0	6.4	8.7	9.3	9.9
1.5 M	5.9	6.2	6.6	8.5	9.0	9.4
2 M	6.1	6.4	6.8	8.3	8.7	9.1
3 M	6.3	6.6	6.9	. 8.2	8.5	
5 M	6.6	6.8	7.0	8.0	8.3	8.5
10 M	6.8	7.0	7.2	7.8	8.0	8.2
20 M	7.0	7.1	7.3	7.7	7.9	8.0
50 M	7.2	7.3	7.3	7.7	7.7	7.8
100 M	7.3	7.3	7.4	7.6	7.7	7.7

Percentage of A's in sample

N	.005	.025	.10	.10	.025	.005
240	2.1	2.6	3.3	7.3	8.6	9.8
260	2.2	2.7	3.4	7.2	8.4	9.6
300	2.3	2.8	3.5	7.0	8.1	9.2
350	2.5	3.0	3.6	6.8	7.8	8.8
400	2.6	3.1	3.7	6.7	7.6	8.5
450	2.7	3.2	3.7	6.6	7.4	8.3
500	2.8	3.3	3.8	6.5	7.3	8.1
600	3.0	3.4	3.9	6.3	7.1	7.8
700	3.1	3.5	4.0	6.2	6.9	7.5
800	3.2	3.6	4.0	6.1	6.8	7.3
1 M	3.4	3.7	4.1	6.0	6.5	7.1
1.5 M	3.7	4.0	4.3	5.8	6.2	6.6
2 M	3.8	4.1	4.4	5.7	6.1	6.4
3 M	4.0	4.2	4.5	5.6	5.8	6.1
5 M	4.2	4.4	4.6	5.4	5.6	5.9
10 M	4.5	4.6	4.7	5.3	5.4	5.6
20 M	4.6	4.7	4.8	5.2	5.3	5.4
50 M	4.8	4.8	4.9	5.1	5.2	5.3
100 M	4.8	4.9	4.9	5.1	5.1	5.2

Note.—*For mode of use see Examples 8 to 12.   M = 1000.   N = total number of individuals
in the sample. P = probability.*

TABLE II—*Continued*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'S IN SAMPLE: 20 AND OVER—*Continued*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Percentage of A's in sample = 3

400	1.2	1.6	2.0	4.4	5.2	5.9
500	1.4	1.7	2.1	4.2	4.9	5.6
600	1.5	1.8	2.1	4.1	4.7	5.3
700	1.6	1.9	2.2	4.0	4.6	5.1
800	1.7	1.9	2.3	3.9	4.4	4.9
1 M	1.8	2.0	2.3	3.8	4.3	4.7
1.5 M	2.0	2.2	2.5	3.6	4.0	4.3
2 M	2.1	2.3	2.5	3.6	3.8	4.1
3 M	2.3	2.4	2.6	3.4	3.7	3.9
5 M	2.4	2.5	2.7	3.3	3.5	3.7
10 M	2.6	2.7	2.8	3.2	3.4	3.5
20 M	2.7	2.8	2.8	3.2	3.2	3.3
50 M	2.8	2.9	2.9	3.1	3.2	3.2
100 M	2.9	2.9	2.9	3.1	3.1	3,1

Percentage of A's in sample = 2

600	.83	1.0	1.3	3.0	3.5	4.0
700	.89	1.1	1.4	2.9	3.3	3.8
800	.96	1.1	1.4	2.8	3.2	3.7
1 M	1.0	1.2	1.5	2.7	3.1	3.4
1.5 M	1.2	1.4	1.6	2.5	2.8	3.1
2 M	1.3	1.4	1.6	2.5	2.7	3.0
3 M	1.4	1.5	1.7	2.4	2.6	2.8
5 M	1.5	1.6	1.8	2.3	2.4	2.6
10 M	1.7	1.7	1.8	2.2	2.3	2.4
20 M	1.8	1.8	1.9	2.1	2.2	2.3
50 M	1.8	1.9	1.9	2.1	2.1	2.2
100 M	1.9	1.9	1.9	2.1	2.1	2.1

Percentage of A's in sample = 1

1 M	.37	.48	.62	1.5	1.8	2.1
1.5 M	.46	.56	.69	1.4	1.6	1.9
2 M	.52	.61	.73	1.4	1.5	1.7
3 M	.59	.68	.78	1.3	1.4	1.6
5 M	.67	.74	.82	1.2	1.3	1.4
7 M	.72	.78	.85	1.2	1.3	1.4
10 M	.76	.81	.87	1.1	1.2	1.3
20 M	.83	.87	.91	1.1	1.1	1.2
50 M	.89	.91	.94	1.1	1.1	1.1
70 M	.91	.93	.95	1.1	1.1	1.1
100 M	.92	.94	.96	1.0	1.1	1.1

Note.—*For mode of use see Examples 8 to 12. M = 1000. N = total number of individuals in the sample. P = probability.*

TABLE II—*Concluded*

CONFIDENCE LIMITS FOR TWOFOLD CLASSIFICATION OF ENUMERATION DATA—
NUMBER OF *A*'s IN SAMPLE: 20 AND OVER—*Concluded*

N\P	Lower limits (%)			Upper limits (%)		
	.005	.025	.10	.10	.025	.005

Percentage of A's in sample = 0.7

N\P	.005	.025	.10	.10	.025	.005
2 M	.31	.38	.47	1.0	1.2	1.3
2.5 M	.34	.41	.50	.97	1.1	1.3
3 M	.37	.43	.51	.94	1.1	1.2
4 M	.41	.47	.54	.90	1.0	1.1
5 M	.43	.49	.55	.88	.97	1.1
7 M	.47	.52	.58	.85	.93	1.0
10 M	.50	.55	.60	.82	.88	.95
20 M	.56	.59	.63	.78	.83	.87
50 M	.61	.63	.65	.75	.78	.80
100 M	.63	.65	.67	.74	.75	.77

Percentage of A's in sample = 0.5

N\P	.005	.025	.10	.10	.025	.005
2.5 M	.21	.26	.33	.73	.86	.99
3 M	.23	.28	.34	.71	.82	.94
4 M	.26	.31	.36	.68	.77	.87
5 M	.28	.32	.38	.65	.74	.82
6 M	.30	.34	.39	.64	.71	.79
7 M	.31	.35	.40	.63	.70	.76
10 M	.34	.37	.41	.60	.66	.71
20 M	.38	.41	.44	.57	.61	.64
50 M	.42	.44	.46	.54	.57	.59
100 M	.44	.46	.47	.53	.55	.56

Percentage of A's in sample = 0.3

N\P	.005	.025	.10	.10	.025	.005
4 M	.12	.16	.20	.44	.52	.60
5 M	.14	.17	.21	.43	.49	.56
6 M	.15	.18	.21	.41	.47	.53
7 M	.16	.19	.22	.40	.46	.51
10 M	.18	.20	.23	.38	.43	.47
20 M	.21	.23	.25	.36	.39	.42
30 M	.22	.24	.26	.34	.37	.39
50 M	.24	.25	.27	.33	.35	.37
70 M	.25	.26	.27	.33	.34	.36
100 M	.26	.27	.28	.32	.34	.35

Percentage of A's in sample = 0.1

N\P	.005	.025	.10	.10	.025	.005
6 M	.026	.033	.053	.18	.22	.26
7 M	.029	.040	.056	.17	.21	.24
10 M	.037	.048	.062	.15	.18	.21
20 M	.052	.061	.073	.14	.15	.17
30 M	.059	.068	.077	.13	.14	.16
50 M	.067	.074	.082	.12	.13	.14
70 M	.072	.078	.085	.12	.13	.14
100 M	.076	.081	.087	.11	.12	.13

Note.—For mode of use see *Examples 8 to 12*. $M = 1000$. N = total number of individuals in the sample. P = probability.

TABLE III

CORRECTION TERMS FOR ESTIMATION OF CONFIDENCE LIMITS—NUMBER OF A's IN SAMPLE GREATER THAN 20; PERCENTAGE OF A's: 10 OR LESS

A's in sample, %	Lower limits			Upper limits		
P	.005	.025	.10	.10	.025	.005
10.0	1.53	.80	.14	1.13	1.73	2.47
9.8	1.54	.80	.14	1.13	1.74	2.48
9.6	1.54	.81	.14	1.14	1.75	2.50
9.4	1.55	.81	.15	1.14	1.76	2.51
9.2	1.56	.82	.15	1.15	1.76	2.53
9.0	1.56	.82	.15	1.15	1.77	2.54
8.8	1.57	.82	.15	1.15	1.78	2.56
8.6	1.58	.83	.16	1.16	1.79	2.57
8.4	1.58	.83	.16	1.16	1.80	2.59
8.2	1.59	.83	.16	1.17	1.81	2.60
8.0	1.60	.84	.16	1.17	1.81	2.61
7.8	1.60	.84	.17	1.17	1.82	2.63
7.6	1.61	.85	.17	1.18	1.83	2.64
7.4	1.62	.85	.17	1.18	1.84	2.66
7.2	1.63	.85	.17	1.19	1.85	2.67
7.0	1.63	.86	.18	1.19	1.86	2.69
6.8	1.64	.86	.18	1.19	1.86	2.70
6.6	1.65	.86	.18	1.20	1.87	2.71
6.4	1.65	.87	.18	1.20	1.88	2.73
6.2	1.66	.87	.19	1.21	1.89	2.74
6.0	1.67	.88	.19	1.21	1.90	2.76
5.8	1.67	.88	.19	1.21	1.91	2.77
5.6	1.68	.88	.19	1.22	1.91	2.79
5.4	1.69	.89	.20	1.22	1.92	2.80
5.2	1.69	.89	.20	1.23	1.93	2.82
5.0	1:70	.90	.20	1.23	1.94	2.83
4.8	1.71	.90	.20	1.23	1.95	2.84
4.6	1.71	.90	.20	1.24	1.96	2.86
4.4	1.72	.91	.21	1.24	1.97	2.87
4.2	1.73	.91	.21	1.25	1.97	2.89
4.0	1.73	.91	.21	1.25	1.98	2.90
3.8	1.74	.92	.21	1.25	1.99	2.92
3.6	1.75	.92	.22	1.26	2.00	2.93
3.4	1.75	.93	.22	1.26	2.01	2.95
3.2	1.76	.93	.22	1.27	2.02	2.96
3.0	1.77	.93	.22	1.27	2.02	2.97
2.8	1.77	.94	.23	1.27	2.03	2.99
2.6	1.78	.94	.23	1.28	2.04	3.00
2.4	1.79	.94	.23	1.28	2.05	3.02
2.2	1.80	.95	.23	1.29	2.06	3.03
2.0	1.80	.95	.24	1.29	2.07	3.05
1.8	1.81	.96	.24	1.29	2.07	3.06
1.6	1.82	.96	.24	1.30	2.08	3.07
1.4	1.82	.96	.24	1.30	2.09	3.09
1.2	1.83	.97	.25	1.31	2.10	3.10
1.0	1.84	.97	.25	1.31	2.11	3.12
0.8	1.84	.97	.25	1.31	2.12	3.13
0.6	1.85	.98	.25	1.32	2.12	3.15
0.4	1.86	.98	.26	1.32	2.13	3.16
0.2	1.86	.99	.26	1.33	2.14	3.18
0.0	1.87	.99	.26	1.33	2.15	3.19

Note.—Produced by interpolation in Table VIIII of Fisher and Yates's Statistical Tables, with permission of the publishers, Messrs. Oliver and Boyd. For mode of use see Example 12. P = probability.

TABLE IV

PROBABILITIES FOR FOURFOLD CONTINGENCY TABLES—
EQUAL SAMPLES UP TO N = 20

Sample No. (1)	(2)	P	Sample No. (1)	(2)	P	Sample No. (1)	(2)	P
N = 1			**N = 7**			**N = 9**		
0 : 1	1 : 0	.5000	0 : 7	7 : 0	.0003	0 : 9	4 : 5	.0412
				6 : 1	.0023		3 : 6	.1030
N = 2				5 : 2	.0105		2 : 7	.2353
0 : 2	2 : 0	.1667		4 : 3	.0350		1 : 8	.5000
	1 : 1	.5000		3 : 4	.0962	1 : 8	8 : 1	.0017
1 : 1	1 : 1	.8333		2 : 5	.2308		7 : 2	.0076
				1 : 6	.5000		6 : 3	.0249
N = 3			1 : 6	6 : 1	.0146		5 : 4	.0656
0 : 3	3 : 0	.0500		5 : 2	.0513		4 : 5	.1471
	2 : 1	.2000		4 : 3	.1329		3 : 6	.2882
	1 : 2	.5000		3 : 4	.2797		2 : 7	.5000
1 : 2	2 : 1	.5000		2 : 5	.5000		1 : 8	.7647
	1 : 2	.8000		1 : 6	.7692	2 : 7	7 : 2	.0283
N = 4			2 : 5	5 : 2	.1431		6 : 3	.0767
0 : 4	4 : 0	.0143		4 : 3	.2960		5 : 4	.1674
	3 : 1	.0714		3 : 4	.5000		4 : 5	.3100
	2 : 2	.2143		2 : 5	.7203		3 : 6	.5000
	1 : 3	.5000	3 : 4	4 : 3	.5000		2 : 7	.7118
1 : 3	3 : 1	.2429		3 : 4	.7040	3 : 6	6 : 3	.1735
	2 : 2	.5000	**N = 8**				5 : 4	.3186
	1 : 3	.7857	0 : 8	8 : 0	.0001		4 : 5	.5000
2 : 2	2 : 2	.7571		7 : 1	.0007		3 : 6	.6900
				6 : 2	.0035	4 : 5	5 : 4	.5000
N = 5				5 : 3	.0128		4 : 5	.6814
0 : 5	5 : 0	.0040		4 : 4	.0385	**N = 10**		
	4 : 1	.0238		3 : 5	.1000	0 : 10	10 : 0	.0000
	3 : 2	.0833		2 : 6	.2333		9 : 1	.0001
	2 : 3	.2222		1 : 7	.5000		8 : 2	.0004
	1 : 4	.5000	1 : 7	7 : 1	.0051		7 : 3	.0015
1 : 4	4 : 1	.1032		6 : 2	.0203		6 : 4	.0054
	3 : 2	.2619		5 : 3	.0594		5 : 5	.0163
	2 : 3	.5000		4 : 4	.1410		4 : 6	.0433
	1 : 4	.7778		3 : 5	.2846		3 : 7	.1053
2 : 3	3 : 2	.5000		2 : 6	.5000		2 : 8	.2369
	2 : 3	.7381		1 : 7	.7667		1 : 9	.5000
N = 6			2 : 6	6 : 2	.0660	1 : 9	9 : 1	.0005
0 : 6	6 : 0	.0011		5 : 3	.1573		8 : 2	.0027
	5 : 1	.0076		4 : 4	.3042		7 : 3	.0099
	4 : 2	.0303		3 : 5	.5000		6 : 4	.0286
	3 : 3	.0909		2 : 6	.7154		5 : 5	.0704
	2 : 4	.2273	3 : 5	5 : 3	.3096		4 : 6	.1517
	1 : 5	.5000		4 : 4	.5000		3 : 7	.2910
1 : 5	5 : 1	.0400		3 : 5	.6958		2 : 8	.5000
	4 : 2	.1212	4 : 4	4 : 4	.6904		1 : 9	.7632
	3 : 3	.2727	**N = 9**			2 : 8	8 : 2	.0115
	2 : 4	.5000	0 : 9	9 : 0	.0000		7 : 3	.0349
	1 : 5	.7727		8 : 1	.0002		6 : 4	.0849
2 : 4	4 : 2	.2836		7 : 2	.0011		5 : 5	.1749
	3 : 3	.5000		6 : 3	.0045		4 : 6	.3143
	2 : 4	.7273		5 : 4	.0147		3 : 7	.5000
3 : 3	3 : 3	.7165					2 : 8	.7090

Note.—For mode of use see Example 14. N = total number of individuals in each sample. P = probability.

TABLE IV—*Continued*

PROBABILITIES FOR FOURFOLD CONTINGENCY TABLES—
EQUAL SAMPLES UP TO N = 20—*Continued*

Sample No. (1)	Sample No. (2)	P
N = 10		
3 : 7	7 : 3	.0894
	6 : 4	.1849
	5 : 5	.3250
	4 : 6	.5000
	3 : 7	.6858
4 : 6	6 : 4	.3281
	5 : 5	.5000
	4 : 6	.6750
5 : 5	5 : 5	.6719
N = 11		
0 : 11	11 : 0	.0000
	10 : 1	.0000
	9 : 2	.0001
	8 : 3	.0005
	7 : 4	.0019
	6 : 5	.0062
	5 : 6	.0175
	4 : 7	.0451
	3 : 8	.1072
	2 : 9	.2381
	1 : 10	.5000
1 : 10	10 : 1	.0002
	9 : 2	.0010
	8 : 3	.0037
	7 : 4	.0119
	6 : 5	.0317
	5 : 6	.0743
	4 : 7	.1554
	3 : 8	.2932
	2 : 9	.5000
	1 : 10	.7619
2 : 9	9 : 2	.0045
	8 : 3	.0150
	7 : 4	.0402
	6 : 5	.0913
	5 : 6	.1807
	4 : 7	.3176
	3 : 8	.5000
	2 : 9	.7068
3 : 8	8 : 3	.0431
	7 : 4	.0992
	6 : 5	.1935
	5 : 6	.3297
	4 : 7	.5000
	3 : 8	.6824
4 : 7	7 : 4	.1974
	6 : 5	.3350
	5 : 6	.5000
	4 : 7	.6703
5 : 6	6 : 5	.5000
	5 : 6	.6650

Sample No. (1)	Sample No. (2)	P
N = 12		
0 : 12	12 : 0	.0000
	10 : 2	.0000
	9 : 3	.0002
	8 : 4	.0007
	7 : 5	.0023
	6 : 6	.0069
	5 : 7	.0186
	4 : 8	.0466
	3 : 9	.1087
	2 : 10	.2391
	1 : 11	.5000
1 : 11	11 : 1	.0001
	10 : 2	.0003
	9 : 3	.0014
	8 : 4	.0047
	7 : 5	.0136
	6 : 6	.0343
	5 : 7	.0775
	4 : 8	.1584
	3 : 9	.2950
	2 : 10	.5000
	1 : 11	.7609
2 : 10	10 : 2	.0017
	9 : 3	.0061
	8 : 4	.0180
	7 : 5	.0447
	6 : 6	.0965
	5 : 7	.1854
	4 : 8	.3202
	3 : 9	.5000
	2 : 10	.7050
3 : 9	9 : 3	.0196
	8 : 4	.0498
	7 : 5	.1069
	6 : 6	.2002
	5 : 7	.3334
	4 : 8	.5000
	3 : 9	.6798
4 : 8	8 : 4	.1102
	7 : 5	.2068
	6 : 6	.3401
	5 : 7	.5000
	4 : 8	.6666
5 : 7	7 : 5	.3421
	6 : 6	.5000
	5 : 7	.6599
6 : 6	6 : 6	.6579
N = 13		
0 : 13	13 : 0	.0000
	11 : 2	.0000
	10 : 3	.0001

Sample No. (1)	Sample No. (2)	P
N = 13		
0 : 13	9 : 4	.0002
	8 : 5	.0008
	7 : 6	.0026
	6 : 7	.0075
	5 : 8	.0196
	4 : 9	.0478
	3 : 10	.1100
	2 : 11	.2400
	1 : 12	.5000
1 : 12	12 : 1	.0000
	11 : 2	.0001
	10 : 3	.0005
	9 : 4	.0018
	8 : 5	.0056
	7 : 6	.0151
	6 : 7	.0365
	5 : 8	.0801
	4 : 9	.1609
	3 : 10	.2965
	2 : 11	.5000
	1 : 12	.7600
2 : 11	11 : 2	.0006
	10 : 3	.0024
	9 : 4	.0077
	8 : 5	.0207
	7 : 6	.0484
	6 : 7	.1008
	5 : 8	.1891
	4 : 9	.3224
	3 : 10	.5000
	2 : 11	.7035
3 : 10	10 : 3	.0085
	9 : 4	.0236
	8 : 5	.0554
	7 : 6	.1131
	6 : 7	.2055
	5 : 8	.3364
	4 : 9	.5000
	3 : 10	.6776
4 : 9	9 : 4	.0576
	8 : 5	.1189
	7 : 6	.2142
	6 : 7	.3441
	5 : 8	.5000
	4 : 9	.6636
5 : 8	8 : 5	.2169
	7 : 6	.3475
	6 : 7	.5000
	5 : 8	.6559
6 : 7	7 : 6	.5000
	6 : 7	.6524

TABLE IV—*Continued*

PROBABILITIES FOR FOURFOLD CONTINGENCY TABLES—
EQUAL SAMPLES UP TO N = 20—*Continued* ·

Sample No. (1)	Sample No. (2)	P	Sample No. (1)	Sample No. (2)	P	Sample No. (1)	Sample No. (2)	P
N = 14			**N = 14**			**N = 15**		
0 : 14	14 : 0	.0000	5 : 9	9 : 5	.1284	3 : 12	9 : 6	.0302
	11 : 3	.0000		8 : 6	.2247		8 : 7	.0641
	10 : 4	.0001		7 : 7	.3518		7 : 8	.1225
	9 : 5	.0003		6 : 8	.5000		6 : 9	.2135
	8 : 6	.0010		5 : 9	.6527		5 : 10	.3408
	7 : 7	.0029	6 : 8	8 : 6	.3532		4 : 11	.5000
	6 : 8	.0080		7 : 7	.5000		3 : 12	.6743
	5 : 9	.0204		6 : 8	.6482	4 : 11	11 : 4	.0134
	4 : 10	.0489	7 : 7	7 : 7	.6468		10 : 5	.0328
	3 : 11	.1111	**N = 15**				9 : 6	.0697
	2 : 12	.2407	0 : 15	15 : 0	.0000		8 : 7	.1318
	1 : 13	.5000		11 : 4	.0000		7 : 8	.2249
1 : 13	13 : 1	.0000		10 : 5	.0001		6 : 9	.3499
	12 : 2	.0000		9 : 6	.0003		5 : 10	.5000
	11 : 3	.0002		8 : 7	.0011		4 : 11	.6592
	10 : 4	.0007		7 : 8	.0032	5 : 10	10 : 5	.0716
	9 : 5	.0022		6 : 9	.0084		9 : 6	.1362
	8 : 6	.0064		5 : 10	.0211		8 : 7	.2311
	7 : 7	.0164		4 : 11	.0498		7 : 8	.3552
	6 : 8	.0384		3 : 12	.1121		6 : 9	.5000
	5 : 9	.0824		2 : 13	.2414		5 : 10	.6501
	4 : 10	.1630		1 : 14	.5000	6 : 9	9 : 6	.2331
	3 : 11	.2978	1 : 14	14 : 1	.0000		8 : 7	.3576
	2 : 12	.5000		13 : 2	.0000		7 : 8	.5000
	1 : 13	.7593		12 : 3	.0001		6 : 9	.6448
2 : 12	12 : 2	.0002		11 : 4	.0002	7 : 8	8 : 7	.5000
	11 : 3	.0009		10 : 5	.0008		7 : 8	.6424
	10 : 4	.0032		9 : 6	.0026			
	9 : 5	.0092		8 : 7	.0071	**N = 16**		
	8 : 6	.0230		7 : 8	.0176	0 : 16	16 : 0	.0000
	7 : 7	.0516		6 : 9	.0400		11 : 5	.0000
	6 : 8	.1043		5 : 10	.0843		10 : 6	.0001
	5 : 9	.1923		4 : 11	.1648		9 : 7	.0004
	4 : 10	.3242		3 : 12	.2988		8 : 8	.0012
	3 : 11	.5000		2 : 13	.5000		7 : 9	.0034
	2 : 12	.7022		1 : 14	.7586		6 : 10	.0088
3 : 11	11 : 3	.0035	2 : 13	13 : 2	.0001		5 : 11	.0217
	10 : 4	.0107		12 : 3	.0003		4 : 12	.0506
	9 : 5	.0271		11 : 4	.0013		3 : 13	.1129
	8 : 6	.0601		10 : 5	.0039		2 : 14	.2419
	7 : 7	.1182		9 : 6	.0105		1 : 15	.5000
	6 : 8	.2099		8 : 7	.0251	1 : 15	15 : 1	.0000
	5 : 9	.3388		7 : 8	.0543		13 : 3	.0000
	4 : 10	.5000		6 : 9	.1074		12 : 4	.0001
	3 : 11	.6758		5 : 10	.1949		11 : 5	.0003
4 : 10	10 : 4	.0285		4 : 11	.3257		10 : 6	.0010
	9 : 5	.0642		3 : 12	.5000		9 : 7	.0030
	8 : 6	.1259		2 : 13	.7011		8 : 8	.0077
	7 : 7	.2200	3 : 12	12 : 3	.0014		7 : 9	.0186
	6 : 8	.3473		11 : 4	.0046		6 : 10	.0415
	5 : 9	.5000		10 : 5	.0127		5 : 11	.0860
	4 : 10	.6612					4 : 12	.1663

TABLE IV—*Continued*

PROBABILITIES FOR FOURFOLD CONTINGENCY TABLES—
EQUAL SAMPLES UP TO N = 20—*Continued*

Sample No. (1)	Sample No. (2)	P
N = 16		
1 : 15	3 : 13	.2998
	2 : 14	.5000
	1 : 15	.7581
2 : 14	14 : 2	.0000
	13 : 3	.0001
	12 : 4	.0005
	11 : 5	.0016
	10 : 6	.0046
	9 : 7	.0117
	8 : 8	.0269
	7 : 9	.0567
	6 : 10	.1100
	5 : 11	.1972
	4 : 12	.3270
	3 : 13	.5000
	2 : 14	.7002
3 : 13	13 : 3	.0005
	12 : 4	.0019
	11 : 5	.0057
	10 : 6	.0145
	9 : 7	.0329
	8 : 8	.0675
	7 : 9	.1262
	6 : 10	.2166
	5 : 11	.3425
	4 : 12	.5000
	3 : 13	.6730
4 : 12	12 : 4	.0061
	11 : 5	.0160
	10 : 6	.0366
	9 : 7	.0744
	8 : 8	.1367
	7 : 9	.2289
	6 : 10	.3521
	5 : 11	.5000
	4 : 12	.6574
5 : 11	11 : 5	.0378
	10 : 6	.0778
	9 : 7	.1426
	8 : 8	.2363
	7 : 9	.3580
	6 : 10	.5000
	5 : 11	.6479
6 : 10	10 : 6	.1445
	9 : 7	.2397
	8 : 8	.3612
	7 : 9	.5000
	6 : 10	.6420
7 : 9	9 : 7	.3622
	8 : 8	.5000
	7 : 9	.6388
8 : 8	8 : 8	.6378

Sample No. (1)	Sample No. (2)	P
N = 17		
0 : 17	17 : 0	.0000
	11 : 6	.0000
	10 : 7	.0001
	9 : 8	.0005
	8 : 9	.0013
	7 : 10	.0036
	6 : 11	.0092
	5 : 12	.0222
	4 : 13	.0513
	3 : 14	.1136
	2 : 15	.2424
	1 : 16	.5000
1 : 16	16 : 1	.0000
	13 : 4	.0000
·	12 : 5	.0001
	11 : 6	.0004
	10 : 7	.0012
	9 : 8	.0033
	8 : 9	.0083
	7 : 10	.0195
	6 : 11	.0427
	5 : 12	.0874
	4 : 13	.1676
	3 : 14	.3006
	2 : 15	.5000
	1 : 16	.7576
2 : 15	15 : 2	.0000
	14 : 3	.0000
	13 : 4	.0002
	12 : 5	.0006
	11 : 6	.0019
	10 : 7	.0052
	9 : 8	.0128
	8 : 9	.0285
	7 : 10	.0588
	6 : 11	.1123
	5 : 12	.1992
	4 : 13	.3281
	3 : 14	.5000
	2 : 15	.6994
3 : 14	14 : 3	.0002
	13 : 4	.0008
	12 : 5	.0024
	11 : 6	.0067
	10 : 7	.0162
	9 : 8	.0354
	8 : 9	.0705
	7 : 10	.1294
	6 : 11	.2192
	5 : 12	.3440
	4 : 13	.5000
	3 : 14	.6719

Sample No. (1)	Sample No. (2)	P
N = 17		
4 : 13	13 : 4	.0026
	12 : 5	.0075
	11 : 6	.0183
	10 : 7	.0399
	9 : 8	.0785
	8 : 9	.1409
	7 : 10	.2323
	6 : 11	.3540
	5 : 12	.5000
	4 : 13	.6560
5 : 12	12 : 5	.0190
	11 : 6	.0422
	10 : 7	.0832
	9 : 8	.1480
	8 : 9	.2406
	7 : 10	.3603
	6 : 11	.5000
	5 : 12	.6460
6 : 11	11 : 6	.0847
	10 : 7	.1514
	9 : 8	.2453
	8 : 9	.3641
	7 : 10	.5000
	6 : 11	.6397
7 : 10	10 : 7	.2468
	9 : 8	.3659
	8 : 9	.5000
	7 : 10	.6358
8 : 9	9 : 8	.5000
	8 : 9	.6341
N = 18		
0 : 18	18 : 0	.0000
	12 : 6	.0000
	11 : 7	.0001
	10 : 8	.0002
	9 : 9	.0005
	8 : 10	.0014
	7 : 11	.0038
	6 : 12	.0095
	5 : 13	.0227
	4 : 14	.0519
	3 : 15	.1143
	2 : 16	.2429
	1 : 17	.5000
1 : 17	17 : 1	.0000
	13 : 5	.0000
	12 : 6	.0001
	11 : 7	.0005
	10 : 8	.0014
	9 : 9	.0036
	8 : 10	.0089

TABLE IV—*Continued*

PROBABILITIES FOR FOURFOLD CONTINGENCY TABLES—
EQUAL SAMPLES UP TO N = 20—*Continued*

Sample No. (1)	Sample No. (2)	P	Sample No. (1)	Sample No. (2)	P	Sample No. (1)	Sample No. (2)	P
N = 18			N = 18			N = 19		
1 : 17	7 : 11	.0204	5 : 13	7 : 11	.3623	2 : 17	13 : 6	.0003
	6 : 12	.0438		6 : 12	.5000		12 : 7	.0009
	5 : 13	.0887		5 : 13	.6444		11 : 8	.0026
	4 : 14	.1688	6 : 12	12 : 6	.0472		10 : 9	.0064
	3 : 15	.3013		11 : 7	.0906		9 : 10	.0146
	2 : 16	.5000		10 : 8	.1573		8 : 11	.0312
	1 : 17	.7571		9 : 9	.2499		7 : 12	.0622
2 : 16	16 : 2	.0000		8 : 10	.3666		6 : 13	.1160
	15 : 3	.0000		7 : 11	.5000		5 : 14	.2024
	14 : 4	.0001		6 : 12	.6377		4 : 15	.3299
	13 : 5	.0002	7 : 11	11 : 7	.1588		3 : 16	.5000
	12 : 6	.0008		10 : 8	.2526		2 : 17	.6981
	11 : 7	.0023		9 : 9	.3690	3 : 16	16 : 3	.0000
	10 : 8	.0058		8 : 10	.5000		15 : 4	.0001
	9 : 9	.0137		7 : 11	.6334		14 : 5	.0004
	8 : 10	.0300	8 : 10	10 : 8	.3697		13 : 6	.0013
	7 : 11	.0606		9 : 9	.5000		12 : 7	.0035
	6 : 12	.1142		8 : 10	.6310		11 : 8	.0085
	5 : 13	.2009	9 : 9	9 : 9	.6302		10 : 9	.0191
	4 : 14	.3291	N = 19				9 : 10	.0394
	3 : 15	.5000	0 : 19	19 : 0	.0000		8 : 11	.0755
	2 : 16	.6987		12 : 7	.0000		7 : 12	.1345
3 : 15	15 : 3	.0001		11 : 8	.0001		6 : 13	.2235
	14 : 4	.0003		10 : 9	.0002		5 : 14	.3464
	13 : 5	.0010		9 : 10	.0006		4 : 15	.5000
	12 : 6	.0030		8 : 11	.0015		3 : 16	.6701
	11 : 7	.0076		7 : 12	.0040	4 : 15	15 : 4	.0005
	10 : 8	.0177		6 : 13	.0098		14 : 5	.0015
	9 : 9	.0375		5 : 14	.0232		13 : 6	.0041
	8 : 10	.0732		4 : 15	.0525		12 : 7	.0101
	7 : 11	.1321		3 : 16	.1149		11 : 8	.0224
	6 : 12	.2215		2 : 17	.2432		10 : 9	.0455
	5 : 13	.3453		1 : 18	.5000		9 : 10	.0852
	4 : 14	.5000	1 : 18	18 : 1	.0000		8 : 11	.1476
	3 : 15	.6709		14 : 5	.0000		7 : 12	.2378
4 : 14	14 : 4	.0011		13 : 6	.0001		6 : 13	.3570
	13 : 5	.0034		12 : 7	.0002		5 : 14	.5000
	12 : 6	.0088		11 : 8	.0005		4 : 15	.6536
	11 : 7	.0205		10 : 9	.0015	5 : 14	14 : 5	.0043
	10 : 8	.0429		9 : 10	.0039		13 : 6	.0109
	9 : 9	.0821		8 : 11	.0094		12 : 7	.0244
	8 : 10	.1445		7 : 12	.0211		11 : 8	.0496
	7 : 11	.2353		6 : 13	.0448		10 : 9	.0918
	6 : 12	.3556		5 : 14	.0899		9 : 10	.1566
	5 : 13	.5000		4 : 15	.1699		8 : 11	.2475
	4 : 14	.6547		3 : 16	.3019		7 : 12	.3640
5 : 13	13 : 5	.0092		2 : 17	.5000		6 : 13	.5000
	12 : 6	.0219		1 : 18	.7568		5 : 14	.6430
	11 : 7	.0461	2 : 17	17 : 2	.0000	6 : 13	13 : 6	.0251
	10 : 8	.0878		15 : 4	.0000		12 : 7	.0516
	9 : 9	.1526		14 : 5	.0001		11 : 8	.0957
	8 : 10	.2443					10 : 9	.1623

TABLE IV—*Concluded*

PROBABILITIES FOR FOURFOLD CONTINGENCY TABLES—
EQUAL SAMPLES UP TO N = 20—*Concluded*

Sample No. (1)	Sample No. (2)	P	Sample No. (1)	Sample No. (2)	P	Sample No. (1)	Sample No. (2)	P
N = 19			**N = 20**			**N = 20**		
6 : 13	9 : 10	.2538	1 : 19	4 : 16	.1708	4 : 16	8 : 12	.1504
	8 : 11	.3687		3 : 17	.3025		7 : 13	.2401
	7 : 12	.5000		2 : 18	.5000		6 : 14	.3582
	6 : 13	.6359		1 : 19	.7564		5 : 15	.5000
7 : 12	12 : 7	.0969	2 : 18	18 : 2	.0000		4 : 16	.6526
	11 : 8	.1650		15 : 5	.0000	5 : 15	15 : 5	.0019
	10 : 9	.2574		14 : 6	.0001		14 : 6	.0052
	9 : 10	.3716		13 : 7	.0004		13 : 7	.0124
	8 : 11	.5000		12 : 8	.0011		12 : 8	.0268
	7 : 12	.6313		11 : 9	.0029		11 : 9	.0527
8 : 11	11 : 8	.2586		10 : 10	.0069		10 : 10	.0954
	10 : 9	.3729		9 : 11	.0155		9 : 11	.1601
	9 : 10	.5000		8 : 12	.0324		8 : 12	.2503
	8 : 11	.6284		7 : 13	.0637		7 : 13·	.3655
9 : 10	10 : 9	.5000		6 : 14	.1176		6 : 14	.5000
	9 : 10	.6271		5 : 15	.2037		5 : 15	.6418
N = 20				4 : 16	.3307	6 : 14	14 : 6	.0128
0 : 20	20 : 0	.0000		3 : 17	.5000		13 : 7	.0281
	12 : 8	.0000		2 : 18	.6975		12 : 8	.0555
	11 : 9	.0001	3 : 17	17 : 3	.0000		11 : 9	.1001
	10 : 10	.0002		16 : 4	.0000		10 : 10	.1666
	9 : 11	.0006		15 : 5	.0002		9 : 11	.2573
	8 : 12	.0016		14 : 6	.0005·		8 : 12	.3705
	7 : 13	.0042		13 : 7	.0015		7 : 13	.5000
	6 : 14	.0101		12 : 8	.0040		6 : 14	.6344
	5 : 15	.0236		11 : 9	.0094	7 : 13	13 : 7	.0564
	4 : 16	.0530		10 : 10	.0204		12 : 8	.1025
	3 : 17	.1154		9 : 11	.0412		11 : 9	.1703
	2 : 18	.2436		8 : 12	.0776		10 : 10	.2615
	1 : 19	.5000		7 : 13	.1367		9 : 11	.3738
1 : 19	19 : 1	.0000		6 : 14	.2253		8 : 12	.5000
	14 : 6	.0000		5 : 15	.3474		7 : 13	.6295
	13 : 7	.0001		4 : 16	.5000	8 : 12	12 : 8	.1715
	12 : 8	.0002		3 : 17	.6693		11 : 9	.2636
	11 : 9	.0006	4 : 16	16 : 4	.0002		10 : 10	.3756
	10 : 10	.0017		15 : 5	.0006		9 : 11	.5000
	9 : 11	.0042		14 : 6	.0018		8 : 12	.6262
	8 : 12	.0098		13 : 7	.0048	9 : 11	11 : 9	.3762
	7 : 13	.0218		12 : 8	.0112		10 : 10	.5000
	6 : 14	.0457		11 : 9	.0242		9 : 11	.6244
	5 : 15	.0909		10 : 10	.0479	10 : 10	10 : 10	.6238
				9 : 11	.0880			

TABLE V

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$

Larger sample (N_1)	Smaller sample (N_2)— minimum differences		
	Highly significant (P < .005)	Significant (P < .025)	

Larger sample (N_1)	Highly significant (P < .005)	Significant (P < .025)
$N_1 = 5$ 0 : 5	**$N_2 = 3$** —	3 : 0 (.0179)
0 : 5	**$N_2 = 4$** —	4 : 0 (.0079)
$N_1 = 6$ 0 : 6	**$N_2 = 3$** —	3 : 0 (.0119)
0 : 6	**$N_2 = 4$** 4 : 0 (.0048)	—
1 : 5	—	4 : 0 (.0238)
0 : 6	**$N_2 = 5$** 5 : 0 (.0022)	4 : 1 (.0152)
1 : 5	—	5 : 0 (.0130)
$N_1 = 7$ 0 : 7	**$N_2 = 3$** —	3 : 0 (.0083)
0 : 7	**$N_2 = 4$** 4 : 0 (.0030)	3 : 1 (.0242)
1 : 6	—	4 : 0 (.0152)
0 : 7	**$N_2 = 5$** 5 : 0 (.0013)	4 : 1 (.0101)
1 : 6	—	5 : 0 (.0076)
0 : 7	**$N_2 = 6$** 5 : 1 (.0047)	4 : 2 (.0210)
1 : 6	6 : 0 (.0040)	—
2 : 5	—	6 : 0 (.0163)
$N_1 = 8$ 0 : 8	**$N_2 = 2$** —	2 : 0 (.0222)
0 : 8	**$N_2 = 3$** —	3 : 0 (.0061)
1 : 7	—	3 : 0 (.0242)
0 : 8	**$N_2 = 4$** 4 : 0 (.0020)	3 : 1 (.0182)
1 : 7	—	4 : 0 (.0101)
0 : 8	**$N_2 = 5$** 5 : 0 (.0008)	4 : 1 (.0070)
1 : 7	5 : 0 (.0047)	—
2 : 6	—	5 : 0 (.0163)
0 : 8	**$N_2 = 6$** 5 : 1 (.0030)	4 : 2 (.0150)
1 : 7	6 : 0 (.0023)	5 : 1 (.0163)
2 : 6	—	6 : 0 (.0093)

Larger sample (N_1)	Highly significant (P < .005)	Significant (P < .025)
$N_1 = 8$ 0 : 8	**$N_2 = 7$** 6 : 1 (.0014)	5 : 2 (.0070)
1 : 7	7 : 0 (.0012)	6 : 1 (.0089)
2 : 6	—	7 : 0 (.0060)
3 : 5	—	7 : 0 (.0187)
$N_1 = 9$ 0 : 9	**$N_2 = 2$** —	2 : 0 (.0182)
0 : 9	**$N_2 = 3$** 3 : 0 (.0045)	—
1 : 8	—	3 : 0 (.0182)
0 : 9	**$N_2 = 4$** 4 : 0 (.0014)	3 : 1 (.0140)
1 : 8	—	4 : 0 (.0070)
2 : 7	—	4 : 0 (.0210)
0 : 9	**$N_2 = 5$** 4 : 1 (.0050−)	—
1 : 8	5 : 0 (.0030)	4 : 1 (.0230)
2 : 7	—	5 : 0 (.0105)
0 : 9	**$N_2 = 6$** 5 : 1 (.0020)	4 : 2 (.0110)
1 : 8	6 : 0 (.0014)	5 : 1 (.0110)
2 : 7	—	6 : 0 (.0060)
3 : 6	—	6 : 0 (.0168)
0 : 9	**$N_2 = 7$** 5 : 2 (.0048)	4 : 3 (.0192)
1 : 8	7 : 0 (.0007)	5 : 2 (.0245)
2 : 7	7 : 0 (.0032)	6 : 1 (.0203)
3 : 6	—	7 : 0 (.0105)
0 : 9	**$N_2 = 8$** 6 : 2 (.0023)	5 : 3 (.0090)
1 : 8	7 : 1 (.0030)	6 : 2 (.0134)
2 : 7	8 : 0 (.0019)	7 : 1 (.0123)
3 : 6	—	8 : 0 (.0067)
4 : 5	—	8 : 0 (.0204)
$N_1 = 10$ 0 : 10	**$N_2 = 2$** —	2 : 0 (.0152)
0 : 10	**$N_2 = 3$** 3 : 0 (.0035)	—
1 : 9	—	3 : 0 (.0140)
0 : 10	**$N_2 = 4$** 4 : 0 (.0010)	3 : 1 (.0110)
1 : 9	4 : 0 (.0050−)	—
2 : 8	—	4 : 0 (.0150)

Note.—*For mode of use see Examples 15 to 18. P = probability (in parentheses).*

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)— minimum differences	
	Highly significant ($P < .005$)	Significant ($P < .025$)
$N_1 = 10$	**$N_2 = 5$**	
0 : 10	4 : 1 (.0037)	3 : 2 (.0219)
1 : 9	5 : 0 (.0020)	4 : 1 (.0170)
2 : 8	—	5 : 0 (.0070)
3 : 7	—	5 : 0 (.0187)
	$N_2 = 6$	
0 : 10	5 : 1 (.0014)	4 : 2 (.0082)
1 : 9	6 : 0 (.0009)	5 : 1 (.0076)
2 : 8	6 : 0 (.0035)	5 : 1 (.0245)
3 : 7	—	6 : 0 (.0105)
	$N_2 = 7$	
0 : 10	5 : 2 (.0034)	4 : 3 (.0147)
1 : 9	6 : 1 (.0037)	5 : 2 (.0176)
2 : 8	7 : 0 (.0019)	6 : 1 (.0134)
3 : 7	—	7 : 0 (.0062)
4 : 6	—	7 : 0 (.0170)
	$N_2 = 8$	
0 : 10	6 : 2 (.0015)	4 : 4 (.0229)
1 : 9	7 : 1 (.0018)	6 : 2 (.0091)
2 : 8	8 : 0 (.0010)	7 : 1 (.0076)
3 : 7	8 : 0 (.0038)	7 : 1 (.0230)
4 : 6	—	8 : 0 (.0113)
	$N_2 = 9$	
0 : 10	6 : 3 (.0031)	5 : 4 (.0108)
1 : 9	7 : 2 (.0049)	6 : 3 (.0174)
2 : 8	8 : 1 (.0045)	7 : 2 (.0186)
3 : 7	9 : 0 (.0024)	8 : 1 (.0149)
4 : 6	—	9 : 0 (.0077)
5 : 5	—	9 : 0 (.0217)
$N_1 = 11$	**$N_2 = 2$**	
0 : 11	—	2 : 0 (.0128)
	$N_2 = 3$	
0 : 11	3 : 0 (.0027)	—
1 : 10	—	3 : 0 (.0110)
	$N_2 = 4$	
0 : 11	4 : 0 (.0007)	3 : 1 (.0088)
1 : 10	4 : 0 (.0037)	—
2 : 9	—	4 : 0 (.0110)
	$N_2 = 5$	
0 : 11	4 : 1 (.0027)	3 : 2 (.0179)
1 : 10	5 : 0 (.0014)	4 : 1 (.0128)
2 : 9	5 : 0 (.0048)	—
3 : 8	—	5 : 0 (.0128)

Larger sample (N_1)	Smaller sample (N_2)— minimum differences	
	Highly significant ($P < .005$)	Significant ($P < .025$)
$N_1 = 11$	**$N_2 = 6$**	
0 : 11	5 : 1 (.0010)	4 : 2 (.0063)
1 : 10	6 : 0 (.0006)	5 : 1 (.0054)
2 : 9	6 : 0 (.0023)	5 : 1 (.0176)
3 : 8	—	6 : 0 (.0067)
4 : 7		6 : 0 (.0170)
	$N_2 = 7$	
0 : 11	5 : 2 (.0025)	4 : 3 (.0114)
1 : 10	6 : 1 (.0024)	5 : 2 (.0129)
2 : 9	7 : 0 (.0011)	6 : 1 (.0091)
3 : 8	7 : 0 (.0038)	6 : 1 (.0249)
4 : 7	—	7 : 0 (.0104)
5 : 6	—	7 : 0 (.0249)
	$N_2 = 8$	
0 : 11	5 : 3 (.0048)	4 : 4 (.0181)
1 : 10	7 : 1 (.0012)	5 : 3 (.0237)
2 : 9	7 : 1 (.0049)	6 : 2 (.0216)
3 : 8	8 : 0 (.0022)	7 : 1 (.0149)
4 : 7	—	8 : 0 (.0065)
5 : 6	—	8 : 0 (.0170)
	$N_2 = 9$	
0 : 11	6 : 3 (.0022)	5 : 4 (.0081)
1 : 10	7 : 2 (.0032)	6 : 3 (.0124)
2 : 9	8 : 1 (.0027)	7 : 2 (.0124)
3 : 8	9 : 0 (.0013)	8 : 1 (.0091)
4 : 7	9 : 0 (.0043)	8 : 1 (.0249)
5 : 6	—	9 : 0 (.0119)
	$N_2 = 10$	
0 : 11	6 : 4 (.0039)	5 : 5 (.0124)
1 : 10	8 : 2 (.0017)	6 : 4 (.0209)
2 : 9	9 : 1 (.0016)	7 : 3 (.0242)
3 : 8	10 : 0 (.0008)	8 : 2 (.0227)
4 : 7	10 : 0 (.0028)	9 : 1 (.0170)
5 : 6	—	10 : 0 (.0085)
5 : 6	—	0 : 10 (.0227)
$N_1 = 12$	**$N_2 = 2$**	
0 : 12	—	2 : 0 (.0110)
	$N_2 = 3$	
0 : 12	3 : 0 (.0022)	—
1 : 11	—	3 : 0 (.0088)
2 : 10	—	3 : 0 (.0219)
	$N_2 = 4$	
0 : 12	4 : 0 (.0005)	3 : 1 (.0071)
1 : 11	4 : 0 (.0027)	—
2 : 10	—	4 : 0 (.0082)
3 : 9	—	4 : 0 (.0192)

TABLE V—Continued

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—Continued

Larger sample (N_1)	Smaller sample (N_2)— minimum differences		Larger sample (N_1)	Smaller sample (N_2)— minimum differences	
	Highly significant (P < .005)	Significant (P < .025)		Highly significant (P < .005)	Significant (P < .025)
$N_1 = 12$ **$N_2 = 5$**			**$N_1 = 12$** **$N_2 = 11$**		
0 : 12	4 : 1 (.0021)	3 : 2 (.0147)	2 : 10	9 : 2 (.0028)	8 : 3 (.0101)
1 : 11	5 : 0 (.0010)	4 : 1 (.0099)	3 : 9	10 : 1 (.0022)	9 : 2 (.0094)
2 : 10	5 : 0 (.0034)	—	4 : 8	11 : 0 (.0010)	10 : 1 (.0070)
3 : 9	—	5 : 0 (.0090)	5 : 7	11 : 0 (.0032)	10 : 1 (.0188)
4 : 8	—	5 : 0 (.0204)	5 : 7	—	0 : 11 (.0235)
			6 : 6	—	11 : 0 (.0092)
$N_2 = 6$			**$N_1 = 13$** **$N_2 = 2$**		
0 : 12	4 : 2 (.0049)	3 : 3 (.0245)	0 : 13	—	2 : 0 (.0095)
1 : 11	5 : 1 (.0040)	4 : 2 (.0217)			
2 : 10	6 : 0 (.0015)	5 : 1 (.0129)	**$N_2 = 3$**		
3 : 9	6 : 0 (.0045)	—	0 : 13	3 : 0 (.0018)	2 : 1 (.0250)
4 : 8	—	6 : 0 (.0113)	1 : 12	—	3 : 0 (.0071)
5 : 7	—	6 : 0 (.0249)	2 : 11	—	3 : 0 (.0179)
$N_2 = 7$			**$N_2 = 4$**		
0 : 12	5 : 2 (.0018)	4 : 3 (.0090)	0 : 13	4 : 0 (.0004)	3 : 1 (.0059)
1 : 11	6 : 1 (.0017)	5 : 2 (.0095)	1 : 12	4 : 0 (.0021)	3 : 1 (.0223)
2 : 10	7 : 0 (.0007)	6 : 1 (.0063)	2 : 11	—	4 : 0 (.0063)
3 : 9	7 : 0 (.0024)	6 : 1 (.0174)	3 : 10	—	4 : 0 (.0147)
4 : 8	—	7 : 0 (.0065)			
5 : 7	—	7 : 0 (.0157)	**$N_2 = 5$**		
			0 : 13	4 : 1 (.0016)	3 : 2 (.0123)
$N_2 = 8$			1 : 12	5 : 0 (.0007)	4 : 1 (.0077)
0 : 12	5 : 3 (.0036)	4 : 4 (.0145)	2 : 11	5 : 0 (.0025)	4 : 1 (.0217)
1 : 11	6 : 2 (.0044)	5 : 3 (.0180)	3 : 10	—	5 : 0 (.0065)
2 : 10	7 : 1 (.0032)	6 : 2 (.0155)	4 : 9	—	5 : 0 (.0147)
3 : 9	8 : 0 (.0013)	7 : 1 (.0099)			
4 : 8	8 : 0 (.0039)	7 : 1 (.0249)	**$N_2 = 6$**		
5 : 7	—	8 : 0 (.0101)	0 : 13	4 : 2 (.0039)	3 : 3 (.0206)
6 : 6	—	8 : 0 (.0238)	1 : 12	5 : 1 (.0029)	4 : 2 (.0173)
			2 : 11	6 : 0 (.0010)	5 : 1 (.0095)
$N_2 = 9$			3 : 10	6 : 0 (.0031)	5 : 1 (.0237)
0 : 12	6 : 3 (.0015)	4 : 5 (.0211)	4 : 9	—	6 : 0 (.0077)
1 : 11	7 : 2 (.0021)	6 : 3 (.0090)	5 : 8	—	6 : 0 (.0170)
2 : 10	8 : 1 (.0017)	7 : 2 (.0085)			
3 : 9	9 : 0 (.0007)	7 : 2 (.0242)	**$N_2 = 7$**		
4 : 8	9 : 0 (.0024)	8 : 1 (.0159)	0 : 13	5 : 2 (.0014)	4 : 3 (.0072)
5 : 7	—	9 : 0 (.0068)	1 : 12	6 : 1 (.0012)	5 : 2 (.0072)
6 : 6	—	9 : 0 (.0170)	2 : 11	6 : 1 (.0044)	5 : 2 (.0223)
			3 : 10	7 : 0 (.0015)	6 : 1 (.0124)
$N_2 = 10$			4 : 9	7 : 0 (.0043)	—
0 : 12	6 : 4 (.0028)	5 : 5 (.0096)	5 : 8	—	7 : 0 (.0101)
1 : 11	7 : 3 (.0047)	6 : 4 (.0155)	6 : 7	—	7 : 0 (.0221)
2 : 10	8 : 2 (.0048)	7 : 3 (.0170)			
3 : 9	9 : 1 (.0035)	8 : 2 (.0150)	**$N_2 = 8$**		
4 : 8	10 : 0 (.0015)	9 : 1 (.0104)	0 : 13	5 : 3 (.0028)	4 : 4 (.0117)
5 : 7	10 : 0 (.0046)	—	1 : 12	6 : 2 (.0032)	5 : 3 (.0139)
6 : 6	—	10 : 0 (.0124)	2 : 11	7 : 1 (.0021)	6 : 2 (.0112)
			3 : 10	8 : 0 (.0008)	7 : 1 (.0067)
$N_2 = 11$			4 : 9	8 : 0 (.0024)	7 : 1 (.0170)
0 : 12	6 : 5 (.0046)	5 : 6 (.0137)	5 : 8	—	8 : 0 (.0063)
1 : 11	8 : 3 (.0025)	6 : 5 (.0239)	6 : 7	—	8 : 0 (.0148)

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant (P < .005)	Significant (P < .025)
$N_1 = 13$	**$N_2 = 9$**	
0 : 13	5 : 4 (.0048)	4 : 5 (.0172)
1 : 12	7 : 2 (.0015)	5 : 4 (.0231)
2 : 11	8 : 1 (.0011)	6 : 3 (.0220)
3 : 10	8 : 1 (.0037)	7 : 2 (.0170)
4 : 9	9 : 0 (.0014)	8 : 1 (.0104)
5 : 8	9 : 0 (.0040)	8 : 1 (.0247)
6 : 7	—	9 : 0 (.0101)
6 : 7	—	0 : 9 (.0230)
	$N_2 = 10$	
0 : 13	6 : 4 (.0021)	4 : 6 (.0237)
1 : 12	7 : 3 (.0033)	6 : 4 (.0116)
2 : 11	8 : 2 (.0032)	7 : 3 (.0122)
3 : 10	9 : 1 (.0022)	8 : 2 (.0101)
4 : 9	10 : 0 (.0009)	9 : 1 (.0066)
5 : 8	10 : 0 (.0026)	9 : 1 (.0167)
6 : 7	—	10 : 0 (.0070)
6 : 7	—	0 : 10 (.0170)
	$N_2 = 11$	
0 : 13	6 : 5 (.0034)	5 : 6 (.0109)
1 : 12	8 : 3 (.0016)	6 : 5 (.0184)
2 : 11	9 : 2 (.0018)	7 : 4 (.0213)
3 : 10	10 : 1 (.0013)	8 : 3 (.0207)
4 : 9	10 : 1 (.0041)	9 : 2 (.0171)
5 : 8	11 : 0 (.0018)	10 : 1 (.0113)
6 : 7	11 : 0 (.0050−)	—
6 : 7	—	0 : 11 (.0128)
	$N_2 = 12$	
0 : 13	7 : 5 (.0016)	5 : 7 (.0149)
1 : 12	8 : 4 (.0033)	7 : 5 (.0100)
2 : 11	9 : 3 (.0041)	8 : 4 (.0127)
3 : 10	10 : 2 (.0038)	9 : 3 (.0131)
4 : 9	11 : 1 (.0027)	10 : 2 (.0114)
5 : 8	12 : 0 (.0012)	11 : 1 (.0080)
5 : 8	—	0 : 12 (.0242)
6 : 7	12 : 0 (.0036)	11 : 1 (.0202)
6 : 7	—	0 : 12 (.0097)
$N_1 = 14$	**$N_2 = 2$**	
0 : 14	—	2 : 0 (.0083)
1 : 13	—	2 : 0 (.0250)
	$N_2 = 3$	
0 : 14	3 : 0 (.0015)	2 : 1 (.0221)
1 : 13	—	3 : 0 (.0059)
2 : 12	—	3 : 0 (.0147)
	$N_2 = 4$	
0 : 14	3 : 1 (.0049)	—
1 : 13	4 : 0 (.0016)	3 : 1 (.0186)

Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant (P < .005)	Significant (P < .025)
$N_1 = 14$	**$N_2 = 4$**	
2 : 12	4 : 0 (.0049)	—
3 : 11	—	4 : 0 (.0114)
4 : 10	—	4 : 0 (.0229)
	$N_2 = 5$	
0 : 14	4 : 1 (.0013)	3 : 2 (.0103)
1 : 13	5 : 0 (.0005)	4 : 1 (.0061)
2 : 12	5 : 0 (.0018)	4 : 1 (.0173)
3 : 11	5 : 0 (.0048)	—
4 : 10	—	5 : 0 (.0108)
5 : 9	—	5 : 0 (.0217)
	$N_2 = 6$	
0 : 14	4 : 2 (.0031)	3 : 3 (.0175)
1 : 13	5 : 1 (.0022)	4 : 2 (.0140)
2 : 12	6 : 0 (.0007)	5 : 1 (.0072)
3 : 11	6 : 0 (.0022)	5 : 1 (.0180)
4 : 10	—	6 : 0 (.0054)
5 : 9	—	6 : 0 (.0119)
6 : 8	—	6 : 0 (.0238)
	$N_2 = 7$	
0 : 14	5 : 2 (.0010)	4 : 3 (.0058)
1 : 13	6 : 1 (.0008)	5 : 2 (.0055)
2 : 12	6 : 1 (.0032)	5 : 2 (.0172)
3 : 11	7 : 0 (.0010)	6 : 1 (.0090)
4 : 10	7 : 0 (.0028)	6 : 1 (.0209)
5 : 9	—	7 : 0 (.0068)
6 : 8	—	7 : 0 (.0148)
	$N_2 = 8$	
0 : 14	5 : 3 (.0021)	4 : 4 (.0096)
1 : 13	6 : 2 (.0023)	5 : 3 (.0109)
2 : 12	7 : 1 (.0015)	6 : 2 (.0084)
3 : 11	7 : 1 (.0047)	6 : 2 (.0220)
4 : 10	8 : 0 (.0015)	7 : 1 (.0119)
5 : 9	8 : 0 (.0040)	—
6 : 8	—	8 : 0 (.0094)
7 : 7	—	8 : 0 (.0201)
	$N_2 = 9$	
0 : 14	5 : 4 (.0037)	4 : 5 (.0142)
1 : 13	6 : 3 (.0049)	5 : 4 (.0183)
2 : 12	7 : 2 (.0042)	6 : 3 (.0166)
3 : 11	8 : 1 (.0025)	7 : 2 (.0122)
4 : 10	9 : 0 (.0009)	8 : 1 (.0070)
5 : 9	9 : 0 (.0025)	8 : 1 (.0167)
6 : 8	—	9 : 0 (.0061)
7 : 7	—	9 : 0 (.0140)
	$N_2 = 10$	
0 : 14	6 : 4 (.0016)	4 : 6 (.0198)
1 : 13	7 : 3 (.0024)	6 : 4 (.0088)

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant (P < .005)	Significant (P < .025)
$N_1 = 14$	**$N_2 = 10$**	
2 : 12	8 : 2 (.0022)	7 : 3 (.0089)
3 : 11	9 : 1 (.0014)	7 : 3 (.0245)
4 : 10	9 : 1 (.0041)	8 : 2 (.0180)
5 : 9	10 : 0 (.0015)	9 : 1 (.0107)
6 : 8	10 : 0 (.0041)	9 : 1 (.0245)
6 : 8	—	0 : 10 (.0223)
7 : 7	—	10 : 0 (.0099)
	$N_2 = 11$	
0 : 14	6 : 5 (.0026)	5 : 6 (.0087)
1 : 13	7 : 4 (.0045)	6 : 5 (.0142)
2 : 12	8 : 3 (.0048)	7 : 4 (.0158)
3 : 11	9 : 2 (.0041)	8 : 3 (.0146)
4 : 10	10 : 1 (.0026)	9 : 2 (.0114)
5 : 9	11 : 0 (.0010)	10 : 1 (.0070)
6 : 8	11 : 0 (.0028)	10 : 1 (.0172)
6 : 8	—	0 : 11 (.0170)
7 : 7	—	11 : 0 (.0071)
	$N_2 = 12$	
0 : 14	6 : 6 (.0040)	5 : 7 (.0120)
1 : 13	8 : 4 (.0023)	6 : 6 (.0209)
2 : 12	9 : 3 (.0027)	8 : 4 (.0091)
3 : 11	10 : 2 (.0024)	9 : 3 (.0089)
4 : 10	11 : 1 (.0016)	9 : 3 (.0236)
5 : 9	11 : 1 (.0047)	10 : 2 (.0187)
6 : 8	12 : 0 (.0019)	11 : 1 (.0121)
6 : 8	—	0 : 12 (.0130)
7 : 7	—	12 : 0 (.0052)
	$N_2 = 13$	
0 : 14	7 : 6 (.0019)	5 : 8 (.0159)
1 : 13	8 : 5 (.0040)	7 : 6 (.0114)
2 : 12	10 : 3 (.0016)	8 : 5 (.0151)
3 : 11	11 : 2 (.0015)	9 : 4 (.0166)
4 : 10	11 : 2 (.0048)	10 : 3 (.0158)
5 : 9	12 : 1 (.0032)	11 : 2 (.0130)
5 : 9	—	0 : 13 (.0248)
6 : 8	13 : 0 (.0014)	12 : 1 (.0087)
6 : 8	—	0 : 13 (.0101)
7 : 7	13 : 0 (.0039)	12 : 1 (.0215)
$N_1 = 15$	**$N_2 = 2$**	
0 : 15	—	2 : 0 (.0074)
1 : 14	—	2 : 0 (.0221)
	$N_2 = 3$	
0 : 15	3 : 0 (.0012)	2 : 1 (.0196)
1 : 14	3 : 0 (.0049)	—
2 : 13	—	3 : 0 (.0123)
3 : 12	—	3 : 0 (.0245)

Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant (P < .005)	Significant (P < .025)
$N_1 = 15$	**$N_2 = 4$**	
0 : 15	3 : 1 (.0041)	—
1 : 14	4 : 0 (.0013)	3 : 1 (.0158)
2 : 13	4 : 0 (.0039)	—
3 : 12	—	4 : 0 (.0090)
4 : 11	—	4 : 0 (.0181)
	$N_2 = 5$	
0 : 15	4 : 1 (.0010)	3 : 2 (.0088)
1 : 14	4 : 1 (.0049)	—
2 : 13	5 : 0 (.0014)	4 : 1 (.0140)
3 : 12	5 : 0 (.0036)	—
4 : 11	—	5 : 0 (.0081)
5 : 10	—	5 : 0 (.0163)
	$N_2 = 6$	
0 : 15	4 : 2 (.0025)	3 : 3 (.0150)
1 : 14	5 : 1 (.0017)	4 : 2 (.0114)
2 : 13	6 : 0 (.0005)	5 : 1 (.0055)
3 : 12	6 : 0 (.0015)	5 : 1 (.0139)
4 : 11	6 : 0 (.0039)	—
5 : 10	—	6 : 0 (.0085)
6 : 9	—	6 : 0 (.0170)
	$N_2 = 7$	
0 : 15	4 : 3 (.0048)	3 : 4 (.0227)
1 : 14	5 : 2 (.0043)	4 : 3 (.0207)
2 : 13	6 : 1 (.0023)	5 : 2 (.0135)
3 : 12	7 : 0 (.0007)	6 : 1 (.0066)
4 : 11	7 : 0 (.0019)	6 : 1 (.0155)
5 : 10	7 : 0 (.0046)	—
6 : 9	—	7 : 0 (.0101)
7 : 8	—	7 : 0 (.0201)
	$N_2 = 8$	
0 : 15	5 : 3 (.0017)	4 : 4 (.0079)
1 : 14	6 : 2 (.0017)	5 : 3 (.0084)
2 : 13	7 : 1 (.0010)	6 : 2 (.0062)
3 : 12	7 : 1 (.0033)	6 : 2 (.0166)
4 : 11	8 : 0 (.0010)	7 : 1 (.0084)
5 : 10	8 : 0 (.0026)	7 : 1 (.0188)
6 : 9	—	8 : 0 (.0061)
7 : 8	—	8 : 0 (.0131)
	$N_2 = 9$	
0 : 15	5 : 4 (.0030)	4 : 5 (.0119)
1 : 14	6 : 3 (.0037)	5 : 4 (.0146)
2 : 13	7 : 2 (.0030)	6 : 3 (.0127)
3 : 12	8 : 1 (.0016)	7 : 2 (.0089)
4 : 11	8 : 1 (.0047)	7 : 2 (.0213)
5 : 10	9 : 0 (.0015)	8 : 1 (.0113)
6 : 9	9 : 0 (.0038)	8 : 1 (.0245)
7 : 8	—	9 : 0 (.0088)
7 : 8	—	0 : 9 (.0186)

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)—minimum differences		Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant $(P < .005)$	Significant $(P < .025)$		Highly significant $(P < .005)$	Significant $(P < .025)$
$N_1 = 15$	$N_2 = 10$		$N_1 = 15$	$N_2 = 14$	
0 : 15	5 : 5 (.0047)	4 : 6 (.0166)	5 : 10	13 : 1 (.0012)	11 : 3 (.0180)
1 : 14	7 : 3 (.0017)	5 : 5 (.0225)	6 : 9	13 : 1 (.0037)	12 : 2 (.0144)
2 : 13	8 : 2 (.0014)	6 : 4 (.0221)	6 : 9	—	0 : 14 (.0105)
3 : 12	8 : 2 (.0048)	7 : 3 (.0181)	7 : 8	14 : 0 (.0015)	13 : 1 (.0095)
4 : 11	9 : 1 (.0027)	8 : 2 (.0127)	7 : 8	0 : 14 (.0041)	1 : 13 (.0225)
5 : 10	10 : 0 (.0009)	9 : 1 (.0070)			
6 : 9	10 : 0 (.0025)	9 : 1 (.0162)	$N_1 = 16$	$N_2 = 2$	
7 : 8	—	10 : 0 (.0060)	0 : 16	—	2 : 0 (.0065)
7 : 8	—	0 : 10 (.0134)	1 : 15	—	2 : 0 (.0196)
	$N_2 = 11$			$N_2 = 3$	
0 : 15	6 : 5 (.0020)	4 : 7 (.0221)	0 : 16	3 : 0 (.0010)	2 : 1 (.0176)
1 : 14	7 : 4 (.0033)	6 : 5 (.0110)	1 : 15	3 : 0 (.0041)	—
2 : 13	8 : 3 (.0035)	7 : 4 (.0119)	2 : 14	—	3 : 0 (.0103)
3 : 12	9 : 2 (.0027)	8 : 3 (.0104)	3 : 13	—	3 : 0 (.0206)
4 : 11	10 : 1 (.0016)	9 : 2 (.0077)			
5 : 10	10 : 1 (.0045)	9 : 2 (.0187)		$N_2 = 4$	
6 : 9	11 : 0 (.0016)	10 : 1 (.0110)	0 : 16	3 : 1 (.0035)	—
6 : 9	—	0 : 11 (.0217)	1 : 15	4 : 0 (.0010)	3 : 1 (.0134)
7 : 8	11 : 0 (.0041)	10 : 1 (.0243)	2 : 14	4 : 0 (.0031)	—
7 : 8	—	0 : 11 (.0098)	3 : 13	—	4 : 0 (.0072)
			4 : 12	—	4 : 0 (.0145)
	$N_2 = 12$				
0 : 15	6 : 6 (.0031)	5 : 7 (.0098)		$N_2 = 5$	
1 : 14	8 : 4 (.0016)	6 : 6 (.0165)	0 : 16	4 : 1 (.0008)	3 : 2 (.0075)
2 : 13	9 : 3 (.0019)	7 : 5 (.0193)	1 : 15	4 : 1 (.0039)	—
3 : 12	10 : 2 (.0016)	8 : 4 (.0192)	2 : 14	5 : 0 (.0010)	4 : 1 (.0114)
4 : 11	10 : 2 (.0048)	9 : 3 (.0166)	3 : 13	5 : 0 (.0028)	—
5 : 10	11 : 1 (.0029)	10 : 2 (.0123)	4 : 12	—	5 : 0 (.0062)
6 : 9	12 : 0 (.0011)	11 : 1 (.0075)	5 : 11	—	5 : 0 (.0124)
6 : 9	—	0 : 12 (.0169)	6 : 10	—	5 : 0 (.0227)
7 : 8	12 : 0 (.0029)	11 : 1 (.0176)			
7 : 8	—	0 : 12 (.0072)		$N_2 = 6$	
			0 : 16	4 : 2 (.0021)	3 : 3 (.0130)
	$N_2 = 13$		1 : 15	5 : 1 (.0013)	4 : 2 (.0093)
0 : 15	6 : 7 (.0046)	5 : 8 (.0131)	2 : 14	5 : 1 (.0043)	—
1 : 14	8 : 5 (.0029)	6 : 7 (.0232)	3 : 13	6 : 0 (.0011)	5 : 1 (.0109)
2 : 13	9 : 4 (.0037)	8 : 5 (.0111)	4 : 12	6 : 0 (.0028)	5 : 1 (.0231)
3 : 12	10 : 3 (.0037)	9 : 4 (.0117)	5 : 11	—	6 : 0 (.0062)
4 : 11	11 : 2 (.0030)	10 : 3 (.0107)	6 : 10	—	6 : 0 (.0124)
5 : 10	12 : 1 (.0019)	11 : 2 (.0083)	7 : 9	—	6 : 0 (.0230)
6 : 9	13 : 0 (.0007)	11 : 2 (.0201)			
6 : 9	—	0 : 13 (.0133)		$N_2 = 7$	
7 : 8	13 : 0 (.0021)	12 : 1 (.0128)	0 : 16	4 : 3 (.0040)	3 : 4 (.0198)
7 : 8	—	0 : 13 (.0054)	1 : 15	5 : 2 (.0034)	4 : 3 (.0172)
			2 : 14	6 : 1 (.0017)	5 : 2 (.0108)
	$N_2 = 14$		3 : 13	6 : 1 (.0049)	—
0 : 15	7 : 7 (.0022)	5 : 9 (.0169)	4 : 12	7 : 0 (.0013)	6 : 1 (.0116)
1 : 14	8 : 6 (.0047)	7 : 7 (.0127)	5 : 11	7 : 0 (.0032)	6 : 1 (.0239)
2 : 13	10 : 4 (.0021)	8 : 6 (.0173)	6 : 10	—	7 : 0 (.0070)
3 : 12	11 : 3 (.0022)	9 : 5 (.0197)	7 : 9	—	7 : 0 (.0140)
4 : 11	12 : 2 (.0019)	10 : 4 (.0198)			

TABLE V—Continued

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—Continued

Larger sample (N_1)	Smaller sample (N_2)— minimum differences	
	Highly significant ($P < .005$)	Significant ($P < .025$)
$N_1 = 16$	**$N_2 = 8$**	
0 : 16	5 : 3 (.0013)	4 : 4 (.0066)
1 : 15	6 : 2 (.0013)	5 : 3 (.0079)
2 : 14	6 : 2 (.0048)	5 : 3 (.0207)
3 : 13	7 : 1 (.0024)	6 : 2 (.0127)
4 : 12	8 : 0 (.0007)	7 : 1 (.0060)
5 : 11	8 : 0 (.0018)	7 : 1 (.0136)
6 : 10	8 : 0 (.0041)	—
7 : 9	—	8 : 0 (.0088)
8 : 8	—	8 : 0 (.0175)
	$N_2 = 9$	
0 : 16	5 : 4 (.0024)	4 : 5 (.0100)
1 : 15	6 : 3 (.0029)	5 : 4 (.0119)
2 : 14	7 : 2 (.0022)	6 : 3 (.0098)
3 : 13	8 : 1 (.0011)	7 : 2 (.0065)
4 : 12	8 : 1 (.0033)	7 : 2 (.0158)
5 : 11	9 : 0 (.0010)	8 : 1 (.0080)
6 : 10	9 : 0 (.0025)	8 : 1 (.0172)
7 : 9	—	9 : 0 (.0056)
7 : 9	—	0 : 9 (.0238)
8 : 8	—	9 : 0 (.0119)
	$N_2 = 10$	
0 : 16	5 : 5 (.0038)	4 : 6 (.0141)
1 : 15	7 : 3 (.0012)	5 : 5 (.0184)
2 : 14	7 : 3 (.0048)	6 : 4 (.0174)
3 : 13	8 : 2 (.0035)	7 : 3 (.0137)
4 : 12	9 : 1 (.0018)	8 : 2 (.0091)
5 : 11	9 : 1 (.0047)	8 : 2 (.0207)
6 : 10	10 : 0 (.0015)	9 : 1 (.0110)
7 : 9	10 : 0 (.0037)	9 : 1 (.0230)
7 : 9	—	0 : 10 (.0174)
8 : 8	—	10 : 0 (.0082)
	$N_2 = 11$	
0 : 16	6 : 5 (.0016)	4 : 7 (.0188)
1 : 15	7 : 4 (.0024)	6 : 5 (.0087)
2 : 14	8 : 3 (.0025)	7 : 4 (.0091)
3 : 13	9 : 2 (.0019)	7 : 4 (.0244)
4 : 12	10 : 1 (.0010)	8 : 3 (.0192)
5 : 11	10 : 1 (.0029)	9 : 2 (.0130)
6 : 10	11 : 0 (.0009)	10 : 1 (.0071)
7 : 9	11 : 0 (.0024)	10 : 1 (.0158)
7 : 9	—	0 : 11 (.0129)
8 : 8	—	11 : 0 (.0058)
	$N_2 = 12$	
0 : 16	6 : 6 (.0025)	4 : 8 (.0242)
1 : 15	7 : 5 (.0043)	6 : 6 (.0132)
2 : 14	8 : 4 (.0048)	7 : 5 (.0149)
3 : 13	9 : 3 (.0043)	8 : 4 (.0141)
4 : 12	10 : 2 (.0032)	9 : 3 (.0117)
5 : 11	11 : 1 (.0018)	10 : 2 (.0083)

Larger sample (N_1)	Smaller sample (N_2)— minimum differences	
	Highly significant ($P < .005$)	Significant ($P < .025$)
$N_1 = 16$	**$N_2 = 12$**	
6 : 10	11 : 1 (.0047)	10 : 2 (.0192)
6 : 10	—	0 : 12 (.0213)
7 : 9	12 : 0 (.0017)	11 : 1 (.0111)
7 : 9	—	0 : 12 (.0097)
8 : 8	12 : 0 (.0041)	11 : 1 (.0241)
	$N_2 = 13$	
0 : 16	6 : 7 (.0036)	5 : 8 (.0108)
1 : 15	8 : 5 (.0022)	6 : 7 (.0187)
2 : 14	9 : 4 (.0026)	7 : 6 (.0228)
3 : 13	10 : 3 (.0025)	8 : 5 (.0234)
4 : 12	12 : 1 (.0003)	9 : 4 (.0217)
5 : 11	12 : 1 (.0011)	10 : 3 (.0180)
6 : 10	12 : 1 (.0031)	11 : 2 (.0131)
6 : 10	—	0 : 13 (.0169)
7 : 9	13 : 0 (.0011)	12 : 1 (.0078)
7 : 9	—	0 : 13 (.0073)
8 : 8	13 : 0 (.0030)	12 : 1 (.0178)
	$N_2 = 14$	
0 : 16	7 : 7 (.0017)	5 : 9 (.0141)
1 : 15	8 : 6 (.0035)	7 : 7 (.0099)
2 : 14	9 : 5 (.0047)	8 : 6 (.0131)
3 : 13	11 : 3 (.0015)	9 : 5 (.0150)
4 : 12	11 : 3 (.0046)	10 : 4 (.0140)
5 : 11	12 : 2 (.0035)	11 : 3 (.0121)
6 : 10	13 : 1 (.0022)	12 : 2 (.0091)
6 : 10	—	0 : 14 (.0135)
7 : 9	14 : 0 (.0008)	12 : 2 (.0213)
7 : 9	—	0 : 14 (.0056)
8 : 8	14 : 0 (.0022)	13 : 1 (.0134)
	$N_2 = 15$	
0 : 16	7 : 8 (.0024)	5 : 10 (.0177)
1 : 15	9 : 6 (.0019)	7 : 8 (.0139)
2 : 14	10 : 5 (.0028)	8 : 7 (.0193)
3 : 13	11 : 4 (.0031)	9 : 6 (.0227)
4 : 12	12 : 3 (.0030)	10 : 5 (.0237)
5 : 11	13 : 2 (.0023)	11 : 4 (.0228)
6 : 10	14 : 1 (.0015)	12 : 3 (.0200)
6 : 10	—	0 : 15 (.0109)
7 : 9	14 : 1 (.0041)	13 : 2 (.0157)
7 : 9	0 : 15 (.0044)	1 : 14 (.0234)
8 : 8	15 : 0 (.0016)	14 : 1 (.0102)
$N_1 = 17$	**$N_2 = 2$**	
0 : 17	—	2 : 0 (.0058)
1 : 16	—	2 : 0 (.0176)
	$N_2 = 3$	
0 : 17	3 : 0 (.0009)	2 : 1 (.0158)
1 : 16	3 : 0 (.0035)	—
2 : 15	—	3 : 0 (.0088)
3 : 14	—	3 : 0 (.0175)

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant $(P < .005)$	Significant $(P < .025)$
$N_1 = 17$	**$N_2 = 4$**	
0 : 17	3 : 1 (.0030)	—
1 : 16	4 : 0 (.0008)	3 : 1 (.0116)
2 : 15	4 : 0 (.0025)	—
3 : 14	—	4 : 0 (.0058)
4 : 13	—	4 : 0 (.0117)
5 : 12	—	4 : 0 (.0211)
	$N_2 = 5$	
0 : 17	4 : 1 (.0007)	3 : 2 (.0065)
1 : 16	4 : 1 (.0032)	3 : 2 (.0239)
2 : 15	5 : 0 (.0008)	4 : 1 (.0093)
3 : 14	5 : 0 (.0021)	4 : 1 (.0207)
4 : 13	5 : 0 (.0048)	—
5 : 12	—	5 : 0 (.0096)
6 : 11	—-	5 : 0 (.0175)
	$N_2 = 6$	
0 : 17	4 : 2 (.0017)	3 : 3 (.0113)
1 : 16	5 : 1 (.0010)	4 : 2 (.0078)
2 : 15	5 : 1 (.0034)	4 : 2 (.0212)
3 : 14	6 : 0 (.0008)	5 : 1 (.0084)
4 : 13	6 : 0 (.0021)	5 : 1 (.0183)
5 : 12	6 : 0 (.0046)	—
6 : 11	—	6 : 0 (.0092)
7 : 10	—	6 : 0 (.0170)
	$N_2 = 7$	
0 : 17	4 : 3 (.0033)	3 : 4 (.0173)
1 : 16	5 : 2 (.0028)	4 : 3 (.0145)
2 : 15	6 : 1 (.0013)	5 : 2 (.0086)
3 : 14	6 : 1 (.0037)	5 : 2 (.0207)
4 : 13	7 : 0 (.0010)	6 : 1 (.0088)
5 : 12	7 : 0 (.0023)	6 : 1 (.0184)
6 : 11	7 : 0 (.0050−)	—
7 : 10	—	7 : 0 (.0099)
8 : 9	—	7 : 0 (.0186)
	$N_2 = 8$	
0 : 17	5 : 3 (.0011)	3 : 5 (.0243)
1 : 16	6 : 2 (.0010)	4 : 4 (.0235)
2 : 15	6 : 2 (.0036)	5 : 3 (.0168)
3 : 14	7 : 1 (.0017)	6 : 2 (.0098)
4 : 13	7 : 1 (.0045)	6 : 2 (.0221)
5 : 12	8 : 0 (.0012)	7 : 1 (.0100)
6 : 11	8 : 0 (.0028)	7 : 1 (.0202)
7 : 10	—	8 : 0 (.0060)
8 : 9	—	8 : 0 (.0119)
8 : 9	—	0 : 8 (.0225)
	$N_2 = 9$	
0 : 17	5 : 4 (.0019)	4 : 5 (.0084)
1 : 16	6 : 3 (.0023)	5 : 4 (.0097)

Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant $(P < .005)$	Significant $(P < .025)$
$N_1 = 17$	**$N_2 = 9$**	
2 : 15	7 : 2 (.0016)	6 : 3 (.0077)
3 : 14	7 : 2 (.0048)	6 : 3 (.0199)
4 : 13	8 : 1 (.0023)	7 : 2 (.0119)
5 : 12	9 : 0 (.0006)	8 : 1 (.0056)
6 : 11	9 : 0 (.0016)	8 : 1 (.0121)
7 : 10	9 : 0 (.0037)	8 : 1 (.0243)
8 : 9	—	9 : 0 (.0078)
8 : 9	—	0 : 9 (.0156)
	$N_2 = 10$	
0 : 17	5 : 5 (.0031)	4 : 6 (.0120)
1 : 16	6 : 4 (.0041)	5 : 5 (.0152)
2 : 15	7 : 3 (.0037)	6 : 4 (.0138)
3 : 14	8 : 2 (.0025)	7 : 3 (.0104)
4 : 13	9 : 1 (.0012)	7 : 3 (.0244)
5 : 12	9 : 1 (.0032)	8 : 2 (.0151)
6 : 11	10 : 0 (.0009)	9 : 1 (.0075)
7 : 10	10 : 0 (.0023)	9 : 1 (.0158)
7 : 10	—	0 : 10 (.0219)
8 : 9	—	10 : 0 (.0052)
8 : 9	—	0 : 10 (.0110)
	$N_2 = 11$	
0 : 17	5 : 6 (.0047)	4 : 7 (.0161)
1 : 16	7 : 4 (.0019)	5 : 6 (.0221)
2 : 15	8 : 3 (.0018)	6 : 5 (.0221)
3 : 14	9 : 2 (.0012)	7 : 4 (.0189)
4 : 13	9 : 2 (.0037)	8 : 3 (.0141)
5 : 12	10 : 1 (.0019)	9 : 2 (.0092)
6 : 11	10 : 1 (.0047)	9 : 2 (.0201)
7 : 10	11 : 0 (.0015)	10 : 1 (.0106)
7 : 10	—	0 : 11 (.0164)
8 : 9	11 : 0 (.0035)	10 : 1 (.0219)
8 : 9	—	0 : 11 (.0078)
	$N_2 = 12$	
0 : 17	6 : 6 (.0019)	4 : 8 (.0208)
1 : 16	7 : 5 (.0032)	6 : 6 (.0106)
2 : 15	8 : 4 (.0036)	7 : 5 (.0116)
3 : 14	9 : 3 (.0031)	8 : 4 (.0106)
4 : 13	10 : 2 (.0021)	9 : 3 (.0084)
5 : 12	11 : 1 (.0011)	9 : 3 (.0197)
6 : 11	11 : 1 (.0030)	10 : 2 (.0131)
7 : 10	12 : 0 (.0010)	11 : 1 (.0071)
7 : 10	—	0 : 12 (.0125)
8 : 9	12 : 0 (.0024)	11 : 1 (.0156)
8 : 9	—	0 : 12 (.0057)
	$N_2 = 13$	
0 : 17	6 : 7 (.0029)	5 : 8 (.0090)
1 : 16	8 : 5 (.0015)	6 : 7 (.0151)
2 : 15.	9 : 4 (.0019)	7 : 6 (.0178)

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)— minimum differences		Larger sample (N_1)	Smaller sample (N_2)— minimum differences	
	Highly significant ($P < .005$)	Significant ($P < .025$)		Highly significant ($P < .005$)	Significant ($P < .025$)
$N_1 = 17$	**$N_2 = 13$**		**$N_1 = 18$**	**$N_2 = 2$**	
3 : 14	10 : 3 (.0017)	8 : 5 (.0179)	0 : 18	—	2 : 0 (.0053)
4 : 13	11 : 2 (.0013)	9 : 4 (.0159)	1 : 17	—	2 : 0 (.0158)
5 : 12	11 : 2 (.0035)	10 : 3 (.0127)			
6 : 11	12 : 1 (.0020)	11 : 2 (.0088)		**$N_2 = 3$**	
6 : 11	—	0 : 13 (.0208)	0 : 18	3 : 0 (.0008)	2 : 1 (.0143)
7 : 10	12 : 1 (.0048)	11 : 2 (.0195)	1 : 17	3 : 0 (.0030)	
7 : 10	—	0 : 13 (.0096)	2 : 16	—	3 : 0 (.0075)
8 : 9	13 : 0 (.0017)	12 : 1 (.0111)	3 : 15	—	3 : 0 (.0150)
8 : 9	0 : 13 (.0042)	1 : 12 (.0238)			
				$N_2 = 4$	
	$N_2 = 14$		0 : 18	3 : 1 (.0026)	—
0 : 17	6 : 8 (.0041)	5 : 9 (.0118)	1 : 17	4 : 0 (.0007)	3 : 1 (.0099)
1 : 16	8 : 6 (.0026)	6 : 8 (.0207)	2 : 16	4 : 0 (.0021)	3 : 1 (.0239)
2 : 15	9 : 5 (.0034)	8 : 6 (.0100)	3 : 15	4 : 0 (.0048)	—
3 : 14	10 : 4 (.0035)	9 : 5 (.0106)	4 : 14	—	4 : 0 (.0096)
4 : 13	11 : 3 (.0031)	10 : 4 (.0099)	5 : 13	—	4 : 0 (.0172)
5 : 12	12 : 2 (.0022)	10 : 4 (.0237)			
6 : 11	13 : 1 (.0012)	11 : 3 (.0192)		**$N_2 = 5$**	
6 : 11	—	0 : 14 (.0168)	0 : 18	4 : 1 (.0005)	3 : 2 (.0056)
7 : 10	13 : 1 (.0033)	12 : 2 (.0137)	1 : 17	4 : 1 (.0027)	3 : 2 (.0208)
7 : 10	—	0 : 14 (.0074)	2 : 16	5 : 0 (.0006)	4 : 1 (.0078)
8 : 9	14 : 0 (.0012)	13 : 1 (.0081)	3 : 15	5 : 0 (.0017)	4 : 1 (.0172)
8 : 9	0 : 14 (.0031)	1 : 13 (.0181)	4 : 14	5 : 0 (.0037)	—
			5 : 13	—	5 : 0 (.0075)
	$N_2 = 15$		6 : 12	—	5 : 0 (.0137)
0 : 17	7 : 8 (.0019)	5 : 10 (.0149)	7 : 11	—	5 : 0 (.0235)
1 : 16	8 : 7 (.0041)	7 : 8 (.0110)			
2 : 15	10 : 5 (.0019)	8 : 7 (.0149)			
3 : 14	11 : 4 (.0021)	9 : 6 (.0170)		**$N_2 = 6$**	
4 : 13	12 : 3 (.0019)	10 : 5 (.0173)	0 : 18	4 : 2 (.0014)	3 : 3 (.0099)
5 : 12	13 : 2 (.0015)	11 : 4 (.0160)	1 : 17	5 : 1 (.0008)	4 : 2 (.0065)
6 : 11	13 : 2 (.0040)	12 : 3 (.0134)	2 : 16	5 : 1 (.0028)	4 : 2 (.0179)
6 : 11	—	0 : 15 (.0137)	3 : 15	6 : 0 (.0006)	5 : 1 (.0079)
7 : 10	14 : 1 (.0024)	13 : 2 (.0098)	4 : 14	6 : 0 (.0016)	5 : 1 (.0146)
7 : 10	—	0 : 15 (.0058)	5 : 13	6 : 0 (.0034)	—
8 : 9	15 : 0 (.0009)	13 : 2 (.0222)	6 : 12	—	6 : 0 (.0069)
8 : 9	0 : 15 (.0023)	1 : 14 (.0139)	7 : 11	—	6 : 0 (.0128)
			8 : 10	—	6 : 0 (.0223)
	$N_2 = 16$				
0 : 17	7 : 9 (.0027)	5 : 11 (.0184)			
1 : 16	9 : 7 (.0021)	7 : 9 (.0149)		**$N_2 = 7$**	
2 : 15	10 : 6 (.0033)	8 : 8 (.0210)	0 : 18	4 : 3 (.0028)	3 : 4 (.0152)
3 : 14	11 : 5 (.0039)	10 : 6 (.0106)	1 : 17	5 : 2 (.0021)	4 : 3 (.0123)
4 : 13	12 : 4 (.0040)	11 : 5 (.0113)	2 : 16	6 : 1 (.0010)	5 : 2 (.0070)
5 : 12	13 : 3 (.0036)	12 : 4 (.0109)	3 : 15	6 : 1 (.0029)	5 : 2 (.0168)
6 : 11	14 : 2 (.0028)	13 : 3 (.0094)	4 : 14	7 : 0 (.0007)	6 : 1 (.0068)
6 : 11	—	0 : 16 (.0112)	5 : 13	7 : 0 (.0016)	6 : 1 (.0142)
7 : 10	15 : 1 (.0017)	13 : 3 (.0218)	6 : 12	7 : 0 (.0036)	—
7 : 10	0 : 16 (.0046)	1 : 15 (.0242)	7 : 11	—	7 : 0 (.0071)
8 : 9	15 : 1 (.0044)	14 : 2 (.0168)	8 : 10	—	7 : 0 (.0134)
8 : 9	0 : 16 (.0018)	1 : 15 (.0107)	9 : 9	—	7 : 0 (.0238)

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)—minimum differences		Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant $(P < .005)$	Significant $(P < .025)$		Highly significant $(P < .005)$	Significant $(P < .025)$
$N_1 = 18$	$N_2 = 8$		$N_1 = 18$	$N_2 = 12$	
0 : 18	4 : 4 (.0047)	3 : 5 (.0215)	0 : 18	6 : 6 (.0016)	4 : 8 (.0181)
1 : 17	5 : 3 (.0045)	4 : 4 (.0201)	1 : 17	7 : 5 (.0025)	6 : 6 (.0086)
2 : 16	6 : 2 (.0028)	5 : 3 (.0138)	2 : 16	8 : 4 (.0026)	7 : 5 (.0091)
3 : 15	7 : 1 (.0012)	6 : 2 (.0077)	3 : 15	9 : 3 (.0022)	7 : 5 (.0241)
4 : 14	7 : 1 (.0033)	6 : 2 (.0174)	4 : 14	10 : 2 (.0020)	8 : 4 (.0197)
5 : 13	8 : 0 (.0008)	7 : 1 (.0074)	5 : 13	10 : 2 (.0039)	9 : 3 (.0150)
6 : 12	8 : 0 (.0019)	7 : 1 (.0151)	6 : 12	11 : 1 (.0020)	10 : 2 (.0091)
7 : 11	8 : 0 (.0041)	—	7 : 11	11 : 1 (.0046)	10 : 2 (.0195)
8 : 10	—	8 : 0 (.0082)	7 : 11	—	0 : 12 (.0156
9 : 9	—	8 : 0 (.0156)	8 : 10	12 : 0 (.0015)	11 : 1 (.0102)
			8 : 10	—	0 : 12 (.0075
	$N_2 = 9$		9 : 9	12 : 0 (.0034)	11 : 1 (.0209)
0 : 18	5 : 4 (.0016)	4 : 5 (.0072)			
1 : 17	6 : 3 (.0017)	5 : 4 (.0080)		$N_2 = 13$	
2 : 16	7 : 2 (.0012)	5 : 4 (.0234)	0 : 18	6 : 7 (.0023)	4 : 9 (.0227)
3 : 15	7 : 2 (.0037)	6 : 3 (.0158)	1 : 17	7 : 6 (.0041)	6 : 7 (.0124)
4 : 14	8 : 1 (.0016)	7 : 2 (.0091)	2 : 16	8 : 5 (.0047)	7 : 6 (.0141)
5 : 13	8 : 1 (.0040)	7 : 2 (.0193)	3 : 15	9 : 4 (.0044)	8 : 5 (.0138)
6 : 12	9 : 0 (.0011)	8 : 1 (.0087)	4 : 14	10 : 3 (.0035)	9 : 4 (.0118)
7 : 11	9 : 0 (.0024)	8 : 1 (.0176)	5 : 13	11 : 2 (.0023)	10 : 3 (.0090)
8 : 10	—	9 : 0 (.0052)	6 : 12	12 : 1 (.0012)	10 : 3 (.0200)
8 : 10	—	0 : 9 (.0197)	7 : 11	12 : 1 (.0030)	11 : 2 (.0132)
9 : 9	—	9 : 0 (.0104)	7 : 11	—	0 : 13 (.0121
			8 : 10	13 : 0 (.0010)	12 : 1 (.0071)
	$N_2 = 10$		8 : 10	—	0 : 13 (.0055
0 : 18	5 : 5 (.0026)	4 : 6 (.0103)	9 : 9	13 : 0 (.0024)	12 : 1 (.0153)
1 : 17	6 : 4 (.0033)	5 : 5 (.0126)			
2 : 16	7 : 3 (.0028)	6 : 4 (.0110)		$N_2 = 14$	
3 : 15	8 : 2 (.0018)	7 : 3 (.0080)	0 : 18	6 : 8 (.0033)	5 : 9 (.0099)
4 : 14	8 : 2 (.0048)	7 : 3 (.0189)	1 : 17	8 : 6 (.0020)	6 : 8 (.0171)
5 : 13	9 : 1 (.0022)	8 : 2 (.0111)	2 : 16	9 : 5 (.0025)	7 : 7 (.0207)
6 : 12	10 : 0 (.0006)	8 : 2 (.0230)	3 : 15	10 : 4 (.0026)	8 : 6 (.0215)
7 : 11	10 : 0 (.0015)	9 : 1 (.0111)	4 : 14	11 : 3 (.0021)	9 : 5 (.0202)
8 : 10	10 : 0 (.0033)	9 : 1 (.0219)	5 : 13	12 : 2 (.0015)	10 : 4 (.0173)
8 : 10	—	0 : 10 (.0141)	6 : 12	12 : 2 (.0039)	11 : 3 (.0134)
9 : 9	—	10 : 0 (.0070)	6 : 12	—	0 : 14 (.0205
			7 : 11	13 : 1 (.0021)	12 : 2 (.0090)
	$N_2 = 11$		7 : 11	—	0 : 14 (.0095
0 : 18	5 : 6 (.0039)	4 : 7 (.0139)	8 : 10	13 : 1 (.0050−)	12 : 2 (.0197)
1 : 17	7 : 4 (.0014)	5 : 6 (.0185)	8 : 10	0 : 14 (.0042)	1 : 13 (.0235
2 : 16	8 : 3 (.0013)	6 : 5 (.0179)	9 : 9	14 : 0 (.0017)	13 : 1 (.0112)
3 : 15	8 : 3 (.0041)	7 : 4 (.0148)			
4 : 14	9 : 2 (.0026)	8 : 3 (.0106)		$N_2 = 15$	
5 : 13	10 : 1 (.0012)	8 : 3 (.0234)	0 : 18	6 : 9 (.0045)	5 : 10 (.0127
6 : 12	10 : 1 (.0031)	9 : 2 (.0144)	1 : 17	8 : 7 (.0031)	6 : 9 (.0226
7 : 11	11 : 0 (.0009)	10 : 1 (.0071)	2 : 16	9 : 6 (.0043)	8 : 7 (.0116
7 : 11	—	0 : 11 (.0204)	3 : 15	10 : 5 (.0047)	9 : 6 (.0129
8 : 10	11 : 0 (.0022)	10 : 1 (.0148)	4 : 14	11 : 4 (.0044)	10 : 5 (.0127
8 : 10	—	0 : 11 (.0102)	5 : 13	12 : 3 (.0036)	11 : 4 (.0113
9 : 9	11 : 0 (.0049)	—	6 : 12	13 : 2 (.0026)	12 : 3 (.0090
			6 : 12	—	0 : 15 (.016
			7 : 11	14 : 1 (.0014)	12 : 3 (.0203

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)—minimum differences		Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant (P < .005)	Significant (P < .025)		Highly significant (P < .005)	Significant (P < .025)
$N_1 = 18$	$N_2 = 15$		$N_1 = 19$	$N_2 = 4$	
7 : 11	—	0 : 15 (.0075)	5 : 14	—	4 : 0 (.0142)
8 : 10	14 : 1 (.0036)	13 : 2 (.0143)	6 : 13	—	4 : 0 (.0237)
8 : 10	0 : 15 (.0032)	1 : 14 (.0183)			
9 : 9	15 : 0 (.0013)	14 : 1 (.0084)		$N_2 = 5$	
			0 : 19	3 : 2 (.0049)	—
	$N_2 = 16$		1 : 18	4 : 1 (.0022)	3 : 2 (.0184)
0 : 18	7 : 9 (.0021)	5 : 11 (.0157)	2 : 17	5 : 0 (.0005)	4 : 1 (.0065)
1 : 17	8 : 8 (.0046)	7 : 9 (.0120)	3 : 16	5 : 0 (.0013)	4 : 1 (.0145)
2 : 16	10 : 6 (.0023)	8 : 8 (.0167)	4 : 15	5 : 0 (.0030)	—
3 : 15	11 : 5 (.0028)	9 : 7 (.0193)	5 : 14	—	5 : 0 (.0059)
4 : 14	12 : 4 (.0027)	10 : 6 (.0204)	6 : 13	—	5 : 0 (.0109)
5 : 13	13 : 3 (.0024)	11 : 5 (.0197)	7 : 12	—	5 : 0 (.0186)
6 : 12	14 : 2 (.0017)	12 : 4 (.0177)			
6 : 12	—	0 : 16 (.0138)		$N_2 = 6$	
7 : 11	14 : 2 (.0044)	13 : 3 (.0145)	0 : 19	4 : 2 (.0012)	3 : 3 (.0087)
7 : 11	—	0 : 16 (.0059)	1 : 18	5 : 1 (.0006)	4 : 2 (.0054)
8 : 10	15 : 1 (.0025)	14 : 2 (.0105)	2 : 17	5 : 1 (.0021)	4 : 2 (.0151)
8 : 10	0 : 16 (.0024)	1 : 15 (.0143)	3 : 16	6 : 0 (.0005)	5 : 1 (.0056)
9 : 9	16 : 0 (.0009)	14 : 2 (.0229)	4 : 15	6 : 0 (.0012)	5 : 1 (.0119)
			5 : 14	6 : 0 (.0026)	5 : 1 (.0225)
	$N_2 = 17$		6 : 13	—	6 : 0 (.0052)
0 : 18	7 : 10 (.0029)	5 : 12 (.0191)	7 : 12	—	6 : 0 (.0097)
1 : 17	9 : 8 (.0025)	7 : 10 (.0159)	8 : 11	—	6 : 0 (.0170)
2 : 16	10 : 7 (.0039)	8 : 9 (.0228)			
3 : 15	11 : 6 (.0048)	10 : 7 (.0122)		$N_2 = 7$	
4 : 14	13 : 4 (.0017)	11 : 6 (.0134)	0 : 19	4 : 3 (.0023)	3 : 4 (.0135)
5 : 13	13 : 4 (0050−)	12 : 5 (.0134)	1 : 18	5 : 2 (.0017)	4 : 3 (.0104)
6 : 12	14 : 3 (.0043)	13 : 4 (.0124)	2 : 17	6 : 1 (.0008)	5 : 2 (.0057)
6 : 12	—	0 : 17 (.0114)	3 : 16	6 : 1 (.0023)	5 : 2 (.0138)
7 : 11	15 : 2 (.0031)	14 : 3 (.0105)	4 : 15	7 : 0 (.0005)	6 : 1 (.0053)
7 : 11	0 : 17 (.0047)	1 : 16 (.0249)	5 : 14	7 : 0 (.0012)	6 : 1 (.0110)
8 : 10	16 : 1 (.0019)	14 : 3 (.0233)	6 : 13	7 : 0 (.0026)	6 : 1 (.0209)
8 : 10	0 : 17 (.0019)	1 : 16 (.0112)	7 : 12	—	7 : 0 (.0052)
9 : 9	16 : 1 (.0047)	15 : 2 (.0178)	8 : 11	—	7 : 0 (.0098)
			9 : 10	—	7 : 0 (.0174)
$N_1 = 19$	$N_2 = 2$				
0 : 19	2 : 0 (.0048)	—		$N_2 = 8$	
1 : 18	—	2 : 0 (.0143)	0 : 19	4 : 4 (.0040)	3 : 5 (.0191)
			1 : 18	5 : 3 (.0037)	4 : 4 (.0172)
	$N_2 = 3$		2 : 17	6 : 2 (.0023)	5 : 3 (.0114)
0 : 19	3 : 0 (.0006)	2 : 1 (.0130)	3 : 16	7 : 1 (.0009)	6 : 2 (.0061)
1 : 18	3 : 0 (.0026)	—	4 : 15	7 : 1 (.0024)	6 : 2 (.0138)
2 : 17	—	3 : 0 (.0065)	5 : 14	8 : 0 (.0006)	7 : 1 (.0056)
3 : 16	—	3 : 0 (.0130)	6 : 13	8 : 0 (.0014)	7 : 1 (.0114)
4 : 15	—	3 : 0 (.0227)	7 : 12	8 : 0 (.0029)	7 : 1 (.0215)
			8 : 11	—	8 : 0 (.0058)
	$N_2 = 4$		9 : 10	—	8 : 0 (.0110)
0 : 19	3 : 1 (.0023)	2 : 2 (.0237)	9 : 10	—	0 : 8 (.0197)
1 : 18	4 : 0 (.0005)	3 : 1 (.0087)			
2 : 17	4 : 0 (.0017)	3 : 1 (.0208)		$N_2 = 9$	
3 : 16	4 : 0 (.0040)	—	0 : 19	5 : 4 (.0013)	4 : 5 (.0062)
4 : 15	—	4 : 0 (.0079)	1 : 18	6 : 3 (.0013)	5 : 4 (.0066)

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant (P < .005)	Significant (P < .025)
$N_1 = 19$	**$N_2 = 9$**	
2 : 17	6 : 3 (.0048)	5 : 4 (.0195)
3 : 16	7 : 2 (.0028)	6 : 3 (.0127)
4 : 15	8 : 1 (.0011)	7 : 2 (.0069)
5 : 14	8 : 1 (.0029)	7 : 2 (.0149)
6 : 13	9 : 0 (.0007)	8 : 1 (.0064)
7 : 12	9 : 0 (.0017)	8 : 1 (.0128)
8 : 11	9 : 0 (.0035)	8 : 1 (.0241)
8 : 11	—	0 : 9 (.0243)
9 : 10	—	9 : 0 (.0070)
9 : 10	—	0 : 9 (.0134)
	$N_2 = 10$	
0 : 19	5 : 5 (.0021)	4 : 6 (.0088)
1 : 18	6 : 4 (.0027)	5 : 5 (.0105)
2 : 17	7 : 3 (.0021)	6 : 4 (.0089)
3 : 16	8 : 2 (.0013)	6 : 4 (.0224)
4 : 15	8 : 2 (.0036)	7 : 3 (.0148)
5 : 14	9 : 1 (.0015)	8 : 2 (.0083)
6 : 13	9 : 1 (.0037)	8 : 2 (.0173)
7 : 12	10 : 0 (.0010)	9 : 1 (.0078)
8 : 11	10 : 0 (.0022)	9 : 1 (.0156)
8 : 11	—	0 : 10 (.0176)
9 : 10	10 : 0 (.0046)	—
9 : 10	—	0 : 10 (.0092)
	$N_2 = 11$	
0 : 19	5 : 6 (.0032)	4 : 7 (.0120)
1 : 18	6 : 5 (.0045)	5 : 6 (.0156)
2 : 17	7 : 4 (.0041)	6 : 5 (.0146)
3 : 16	8 : 3 (.0031)	7 : 4 (.0115)
4 : 15	9 : 2 (.0019)	8 : 3 (.0080)
5 : 14	9 : 2 (.0047)	8 : 3 (.0179)
6 : 13	10 : 1 (.0022)	9 : 2 (.0105)
7 : 12	10 : 1 (.0048)	9 : 2 (.0213)
7 : 12	—	0 : 11 (.0248)
8 : 11	11 : 0 (.0014)	10 : 1 (.0102)
8 : 11	—	0 : 11 (.0129)
9 : 10	11 : 0 (.0031)	10 : 1 (.0200)
9 : 10	—	0 : 11 (.0065)
	$N_2 = 12$	
0 : 19	5 : 7 (.0047)	4 : 8 (.0157)
1 : 18	7 : 5 (.0020)	5 : 7 (.0217)
2 : 17	8 : 4 (.0020)	6 : 6 (.0220)
3 : 16	9 : 3 (.0016)	7 : 5 (.0193)
4 : 15	9 : 3 (.0044)	8 : 4 (.0152)
5 : 14	10 : 2 (.0028)	9 : 3 (.0106)
6 : 13	11 : 1 (.0012)	9 : 3 (.0227)
7 : 12	11 : 1 (.0030)	10 : 2 (.0137)
7 : 12	—	0 : 12 (.0192)
8 : 11	12 : 0 (.0009)	11 : 1 (.0068)
8 : 11	—	0 : 12 (.0096)

Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant (P < .005)	Significant (P < .025)
$N_1 = 19$	**$N_2 = 12$**	
9 : 10	12 : 0 (.0021)	11 : 1 (.0140)
9 : 10	0 : 12 (.0046)	—
	$N_2 = 13$	
0 : 19	6 : 7 (.0019)	4 : 9 (.0199)
1 : 18	7 : 6 (.0032)	6 : 7 (.0102)
2 : 17	8 : 5 (.0036)	7 : 6 (.0114)
3 : 16	9 : 4 (.0033)	8 : 5 (.0106)
4 : 15	10 : 3 (.0026)	9 : 4 (.0088)
5 : 14	11 : 2 (.0016)	9 : 4 (.0202)
6 : 13	11 : 2 (.0040)	10 : 3 (.0145)
7 : 12	12 : 1 (.0020)	11 : 2 (.0090)
7 : 12	—	0 : 13 (.0150)
8 : 11	12 : 1 (.0046)	11 : 2 (.0190)
8 : 11	—	0 : 13 (.0072)
9 : 10	13 : 0 (.0014)	12 : 1 (.0099)
9 : 10	0 : 13 (.0033)	1 : 12 (.0200)
	$N_2 = 14$	
0 : 19	6 : 8 (.0027)	4 : 10 (.0245)
1 : 18	7 : 7 (.0049)	6 : 8 (.0142)
2 : 17	9 : 5 (.0019)	7 : 7 (.0168)
3 : 16	10 : 4 (.0018)	8 : 6 (.0169)
4 : 15	11 : 3 (.0015)	9 : 5 (.0153)
5 : 14	11 : 3 (.0039)	10 : 4 (.0127)
6 : 13	12 : 2 (.0026)	11 : 3 (.0094)
6 : 13	—	0 : 14 (.0245)
7 : 12	13 : 1 (.0012)	11 : 3 (.0203)
7 : 12	—	0 : 14 (.0118)
8 : 11	13 : 1 (.0031)	12 : 2 (.0132)
8 : 11	—	0 : 14 (.0054)
9 : 10	14 : 0 (.0010)	13 : 1 (.0071)
9 : 10	0 : 14 (.0024)	1 : 13 (.0150)
	$N_2 = 15$	
0 : 19	6 : 9 (.0037)	5 : 10 (.0108)
1 : 18	8 : 7 (.0024)	6 : 9 (.0189)
2 : 17	9 : 6 (.0032)	7 : 8 (.0234)
3 : 16	10 : 5 (.0034)	8 : 7 (.0250−)
4 : 15	11 : 4 (.0031)	9 : 6 (.0243)
5 : 14	12 : 3 (.0024)	10 : 5 (.0219)
6 : 13	13 : 2 (.0016)	11 : 4 (.0183)
6 : 13	—	0 : 15 (.0202)
7 : 12	13 : 2 (.0041)	12 : 3 (.0140)
7 : 12	—	0 : 15 (.0094)
8 : 11	14 : 1 (.0022)	13 : 2 (.0094)
8 : 11	0 : 15 (.0042)	1 : 14 (.0234)
9 : 10	15 : 0 (.0007)	13 : 2 (.0199)
9 : 10	0 : 15 (.0018)	1 : 14 (.0113)
	$N_2 = 16$	
0 : 19	6 : 10 (.0049)	5 : 11 (.0135)
1 : 18	8 : 8 (.0037)	6 : 10 (.0243)

TABLE V—Continued

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—Continued

Larger sample (N_1)	Smaller sample (N_2)— minimum differences		Larger sample (N_1)	Smaller sample (N_2)— minimum differences	
	Highly significant (P < .005)	Significant (P < .025)		Highly significant (P < .005)	Significant (P < .025)
$N_1 = 19$	**$N_2 = 16$**		**$N_1 = 20$**	**$N_2 = 3$**	
2 : 17	10 : 6 (.0017)	8 : 8 (.0132)	2 : 18	—	3 : 0 (.0056)
3 : 16	11 : 5 (.0019)	9 : 7 (.0150)	3 : 17	—	3 : 0 (.0113)
4 : 15	12 : 4 (.0018)	10 : 6 (.0153)	4 : 16	—	3 : 0 (.0198)
5 : 14	13 : 3 (.0015)	11 : 5 (.0143)			
6 : 13	13 : 3 (.0040)	12 : 4 (.0124)		**$N_2 = 4$**	
6 : 13	—	0 : 16 (.0167)	0 : 20	3 : 1 (.0020)	2 : 2 (.0217)
7 : 12	14 : 2 (.0028)	13 : 3 (.0098)	1 : 19	4 : 0 (.0005)	3 : 1 (.0076)
7 : 12	—	0 : 16 (.0075)	2 : 18	4 : 0 (.0014)	3 : 1 (.0184)
8 : 11	15 : 1 (.0015)	13 : 3 (.0210)	3 : 17	4 : 0 (.0033)	—
8 : 11	0 : 16 (.0032)	1 : 15 (.0184)	4 : 16	—	4 : 0 (.0066)
9 : 10	15 : 1 (.0037)	14 : 2 (.0148)	5 : 15	—	4 : 0 (.0119)
9 : 10	0 : 16 (.0013)	1 : 15 (.0086)	6 : 14	—	4 : 0 (.0198)
	$N_2 = 17$			**$N_2 = 5$**	
0 : 19	7 : 10 (.0023)	5 : 12 (.0164)	0 : 20	3 : 2 (.0043)	—
1 : 18	9 : 8 (.0019)	7 : 10 (.0130)	1 : 19	4 : 1 (.0019)	3 : 2 (.0162)
2 : 17	10 : 7 (.0029)	8 : 9 (.0183)	2 : 18	5 : 0 (.0004)	4 : 1 (.0054)
3 : 16	11 : 6 (.0035)	9 : 8 (.0217)	3 : 17	5 : 0 (.0011)	4 : 1 (.0122)
4 : 15	12 : 5 (.0036)	10 : 7 (.0234)	4 : 16	5 : 0 (.0024)	4 : 1 (.0235)
5 : 14	13 : 4 (.0034)	11 : 6 (.0233)	5 : 15	5 : 0 (.0047)	—
6 : 13	14 : 3 (.0027)	12 : 5 (.0219)	6 : 14	—	5 : 0 (.0087)
6 : 13	—	0 : 17 (.0139)	7 : 13	—	5 : 0 (.0149)
7 : 12	15 : 2 (.0019)	13 : 4 (.0191)	8 : 12	—	5 : 0 (.0242)
7 : 12	—	0 : 17 (.0060)			
8 : 11	15 : 2 (.0048)	14 : 3 (.0154)		**$N_2 = 6$**	
8 : 11	0 : 17 (.0025)	1 : 16 (.0146)	0 : 20	4 : 2 (.0010)	3 : 3 (.0077)
9 : 10	16 : 1 (.0027)	15 : 2 (.0110)	1 : 19	4 : 2 (.0047)	—
9 : 10	0.17 (.0010)	2 : 15 (.0236)	2 : 18	5 : 1 (.0017)	4 : 2 (.0129)
			3 : 17	5 : 1 (.0045)	
	$N_2 = 18$		4 : 16	6 : 0 (.0009)	5 : 1 (.0097)
0 : 19	7 : 11 (.0031)	5 : 13 (.0197)	5 : 15	6 : 0 (.0020)	5 : 1 (.0184)
1 : 18	9 : 9 (.0028)	7 : 11 (.0168)	6 : 14	6 : 0 (.0040)	—
2 : 17	10 : 8 (.0043)	8 : 10 (.0243)	7 : 13	—	6 : 0 (.0075)
3 : 16	12 : 6 (.0021)	10 : 8 (.0135)	8 : 12	—	6 : 0 (.0130)
4 : 15	13 : 5 (.0023)	11 : 7 (.0153)	9 : 11	—	6 : 0 (.0217)
5 : 14	14 : 4 (.0022)	12 : 6 (.0159)			
6 : 13	15 : 3 (.0018)	13 : 5 (.0154)		**$N_2 = 7$**	
6 : 13	—	0 : 18 (.0117)	0 : 20	4 : 3 (.0020)	3 : 4 (.0120)
7 : 12	15 : 3 (.0048)	14 : 4 (.0138)	1 : 19	5 : 2 (.0014)	4 : 3 (.0090)
7 : 12	0 : 18 (.0049)	—	2 : 18	5 : 2 (.0047)	4 : 3 (.0239)
8 : 11	16 : 2 (.0035)	15 : 3 (.0115)	3 : 17	6 : 1 (.0017)	5 : 2 (.0114)
8 : 11	0 : 18 (.0020)	1 : 17 (.0116)	4 : 16	6 : 1 (.0041)	5 : 2 (.0234)
9 : 10	17 : 1 (.0020)	15 : 3 (.0247)	5 : 15	7 : 0 (.0009)	6 : 1 (.0087)
9 : 10	0 : 18 (.0007)	2 : 16 (.0185)	6 : 14	7 : 0 (.0019)	6 : 1 (.0165)
			7 : 13	7 : 0 (.0039)	
$N_1 = 20$	**$N_2 = 2$**		8 : 12	—	7 : 0 (.0072)
0 : 20	2 : 0 (.0043)	—	9 : 11	—	7 : 0 (.0129)
1 : 19	—	2 : 0 (.0130)	10 : 10	—	7 : 0 (.0219)
	$N_2 = 3$			**$N_2 = 8$**	
0 : 20	3 : 0 (.0003)	2 : 1 (.0119)	0 : 20	4 : 4 (.0034)	3 : 5 (.0171)
1 : 19	3 : 0 (.0023)	—	1 : 19	5 : 3 (.0031)	4 : 4 (.0148)

TABLE V—*Continued*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Continued*

Larger sample (N_1)	Smaller sample (N_2)— minimum differences		Larger sample (N_1)	Smaller sample (N_2)— minimum differences	
	Highly significant (P < .005)	Significant (P < .025)		Highly significant (P < .005)	Significant (P < .025)
$N_1 = 20$	**$N_2 = 8$**		**$N_1 = 20$**	**$N_2 = 11$**	
2 : 18	6 : 2 (.0018)	5 : 3 (.0095)	9 : 11	11 : 0 (.0020)	10 : 1 (.0140)
3 : 17	6 : 2 (.0048)	5 : 3 (.0223)	9 : 11	—	0 : 11 (.0083)
4 : 16	7 : 1 (.0019)	6 : 2 (.0110)	10 : 10	11 : 0 (.0042)	—
5 : 15	7 : 1 (.0043)	6 : 2 (.0221)			
6 : 14	8 : 0 (.0010)	7 : 1 (.0087)		**$N_2 = 12$**	
7 : 13	8 : 0 (.0021)	7 : 1 (.0164)	0 : 20	5 : 7 (.0039)	4 : 8 (.0138)
·8 : 12	8 : 0 (.0041)	—	1 : 19	7 : 5 (.0015)	5 : 7 (.0185)
9 : 11	—	8 : 0 (.0078)	2 : 18	8 : 4 (.0016)	6 : 6 (.0182)
9 : 11	—	0 : 8 (.0243)	3 : 17	8 : 4 (.0047)	7 : 5 (.0156)
10 : 10	—	8 : 0 (.0141)	4 : 16	9 : 3 (.0033)	8 : 4 (.0118)
			5 : 15	10 : 2 (.0019)	9 : 3 (.0079)
	$N_2 = 9$		6 : 14	10 : 2 (.0046)	9 : 3 (.0170)
0 : 20	5 : 4 (.0011)	3 : 6 (.0230)	7 : 13	11 : 1 (.0021)	10 : 2 (.0098)
1 : 19	6 : 3 (.0011)	4 : 5 (.0223)	7 : 13	—	0.12 (.0230)
2 : 18	6 : 3 (.0039)	5 : 4 (.0164)	8 : 12	11 : 1 (.0046)	10 : 2 (.0197)
3 : 17	7 : 2 (.0021)	6 : 3 (.0103)	8 : 12	—	0 : 12 (.0120)
4 : 16	8 : 1 (.0008)	6 : 3 (.0224)	9 : 11	12 : 0 (.0013)	11 : 1 (.0095)
5 : 15	8 : 1 (.0022)	7 : 2 (.0116)	9 : 11	—	0 : 12 (.0060)
6 : 14	8 : 1 (.0047)	7 : 2 (.0228)	10 : 10	12 : 0 (.0029)	11 : 1 (.0185)
7 : 13	9 : 0 (.0011)	8 : 1 (.0095)			
8 : 12	9 : 0 (.0024)	8 : 1 (.0178)		**$N_2 = 13$**	
9 : 11	9 : 0 (.0049)	—	0 : 20	6 : 7 (.0016)	4 : 9 (.0175)
9 : 11	—	0 : 9 (.0168)	1 : 19	7 : 6 (.0026)	5 : 8 (.0248)
10 : 10	—	9 : 0 (.0092)	2 : 18	8 : 5 (.0028)	7 : 6 (.0092)
			3 : 17	9 : 4 (.0025)	7 : 6 (.0239)
	$N_2 = 10$		4 : 16	10 : 3 (.0018)	8 : 5 (.0201)
0 : 20	5 : 5 (.0018)	4 : 6 (.0077)	5 : 15	10 : 3 (.0047)	9 : 4 (.0153)
1 : 19	6 : 4 (.0022)	5 : 5 (.0089)	6 : 14	11 : 2 (.0028)	10 : 3 (.0106)
2 : 18	7 : 3 (.0017)	6 : 4 (.0072)	7 : 13	12 : 1 (.0012)	10 : 3 (.0218)
3 : 17	7 : 3 (.0049)	6 : 4 (.0184)	7 : 13	—	0 : 13 (.0181)
4 : 16	8 : 2 (.0026)	7 : 3 (.0115)	8 : 12	12 : 1 (.0030)	11 : 2 (.0132)
5 : 15	9 : 1 (.0011)	7 : 3 (.0241)	8 : 12	—	0 : 13 (.0091)
6 : 14	9 : 1 (.0026)	8 : 2 (.0131)	9 : 11	13 : 0 (.0009)	12 : 1 (.0066)
7 : 13	10 : 0 (.0006)	9 : 1 (.0056)	9 : 11	0 : 13 (.0044)	—
8 : 12	10 : 0 (.0015)	9 : 1 (.0111)	10 : 10	13 : 0 (.0020)	12 : 1 (.0133)
8 : 12	—	0 : 10 (.0215)			
9 : 11	10 : 0 (.0031)	9 : 1 (.0209)		**$N_2 = 14$**	
9 : 11	—	0 : 10 (.0117)	0 : 20	6 : 8 (.0022)	4 : 10 (.0216)
10 : 10	—	10 : 0 (.0061)	1 : 19	7 : 7 (.0040)	6 : 8 (.0118)
			2 : 18	8 : 6 (.0047)	7 : 7 (.0135)
	$N_2 = 11$		3 : 17	9 : 5 (.0045)	8 : 6 (.0134)
0 : 20	5 : 6 (.0027)	4 : 7 (.0105)	4 : 16	10 : 4 (.0038)	9 : 5 (.0118)
1 : 19	6 : 5 (.0036)	5 : 6 (.0132)	5 : 15	11 : 3 (.0028)	10 : 4 (.0094)
2 : 18	7 : 4 (.0033)	6 : 5 (.0119)	6 : 14	12 : 2 (.0017)	10 : 4 (.0204)
3 : 17	8 : 3 (.0023)	7 : 4 (.0092)	7 : 13	12 : 2 (.0041)	11 : 3 (.0145)
4 : 16	9 : 2 (.0014)	7 : 4 (.0212)	7 : 13	—	0 : 14 (.0144)
5 : 15	9 : 2 (.0034)	8 : 3 (.0138)	8 : 12	13 : 1 (.0020)	12 : 2 (.0090)
6 : 14	10 : 1 (.0015)	9 : 2 (.0077)	8 : 12	—	0 : 14 (.0069)
7 : 13	10 : 1 (.0033)	9 : 2 (.0157)	9 : 11	13 : 1 (.0045)	12 : 2 (.0185)
8 : 12	11 : 0 (.0009)	10 : 1 (.0071)	9 : 11	0 : 14 (.0032)	1 : 13 (.0193)
8 : 12	—	0 : 11 (.0160)	10 : 10	14 : 0 (.0014)	13 : 1 (.0096)

TABLE V—*Concluded*

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES—
UNEQUAL SAMPLES UP TO $N_1 = 20$, $N_2 = 19$—*Concluded*

Larger sample (N_1)	Smaller sample (N_2)—minimum differences		Larger sample (N_1)	Smaller sample (N_2)—minimum differences	
	Highly significant (P < .005)	Significant (P < .025)		Highly significant (P < .005)	Significant (P < .025)
$N_1 = 20$	**$N_2 = 15$**		**$N_1 = 20$**	**$N_2 = 17$**	
0 : 20	6 : 9 (.0031)	5 : 10 (.0093)	7 : 13	14 : 3 (.0045)	13 : 4 (.0134)
1 : 19	8 : 7 (.0019)	6 : 9 (.0159)	7 : 13	—	0 : 17 (.0075)
2 : 18	9 : 6 (.0024)	7 : 8 (.0192)	8 : 12	15 : 2 (.0030)	14 : 3 (.0103)
3 : 17	10 : 5 (.0025)	8 : 7 (.0200)	8 : 12	0 : 17 (.0033)	1 : 16 (.0186)
4 : 16	11 : 4 (.0022)	9 : 6 (.0189)	9 : 11	16 : 1 (.0016)	14 : 3 (.0217)
5 : 15	12 : 3 (.0017)	10 : 5 (.0165)	9 : 11	0 : 17 (.0014)	1 : 16 (.0087)
6 : 14	12 : 3 (.0043)	11 : 4 (.0134)	10 : 10	16 : 1 (.0039)	15 : 2 (.0152)
6 : 14	—	0 : 15 (.0239)			
7 : 13	13 : 2 (.0027)	12 : 3 (.0098)		**$N_2 = 18$**	
7 : 13	—	0 : 15 (.0115)	0 : 20	7 : 11 (.0025)	5 : 13 (.0171)
8 : 12	14 : 1 (.0014)	12 : 3 (.0204)	1 : 19	9 : 9 (.0022)	7 : 11 (.0139)
8 : 12	—	0 : 15 (.0054)	2 : 18	10 : 8 (.0033)	8 : 10 (.0198)
9 : 11	14 : 1 (.0032)	13 : 2 (.0134)	3 : 17	11 : 7 (.0042)	9 : 9 (.0238)
9 : 11	0 : 15 (.0024)	1 : 14 (.0147)	4 : 16	12 : 6 (.0044)	11 : 7 (.0115)
10 : 10	15 : 0 (.0010)	14 : 1 (.0070)	5 : 15	13 : 5 (.0044)	12 : 6 (.0115)
			6 : 14	14 : 4 (.0039)	13 : 5 (.0109)
	$N_2 = 16$		6 : 14	—	0 : 18 (.0140)
0 : 20	6 : 10 (.0041)	5 : 11 (.0116)	7 : 13	15 : 3 (.0031)	13 : 5 (.0237)
1 : 19	8 : 8 (.0028)	6 : 10 (.0206)	7 : 13	—	0 : 18 (.0061)
2 : 18	9 : 7 (.0039)	8 : 8 (.0105)	8 : 12	16 : 2 (.0021)	14 : 4 (.0204)
3 : 17	10 : 6 (.0044)	9 : 7 (.0117)	8 : 12	0.18 (.0026)	1 : 17 (.0149)
4 : 16	11 : 5 (.0042)	10 : 6 (.0116)	9 : 11	17 : 1 (.0011)	15 : 3 (.0163)
5 : 15	12 : 4 (.0036)	11 : 5 (.0106)	9 : 11	0 : 18 (.0010)	2 : 16 (.0243)
6 : 14	13 : 3 (.0027)	11 : 5 (.0233)	10 : 10	17 : 1 (.0029)	16 : 2 (.0115)
6 : 14	—	0 : 16 (.0199)			
7 : 13	14 : 2 (.0018)	12 : 4 (.0191)		**$N_2 = 19$**	
7 : 13	—	0 : 16 (.0093)	0 : 20	7 : 12 (.0033)	5 : 14 (.0202)
8 : 12	14 : 2 (.0043)	13 : 3 (.0145)	1 : 19	9 : 10 (.0030)	7 : 12 (.0176)
8 : 12	0 : 16 (.0042)	1 : 15 (.0232)	2 : 18	10 : 9 (.0049)	9 : 10 (.0116)
9 : 11	15 : 1 (.0022)	14 : 2 (.0096)	3 : 17	12 : 7 (.0025)	10 : 9 (.0149)
9 : 11	0 : 16 (.0018)	1 : 15 (.0113)	4 : 16	13 : 6 (.0029)	11 : 8 (.0171)
10 : 10	16 : 0 (.0007)	14 : 2 (.0199)	5 : 15	14 : 5 (.0029)	12 : 7 (.0182)
			6 : 14	15 : 4 (.0027)	13 : 6 (.0182)
	$N_2 = 17$		6 : 14	—	0 : 19 (.0119)
0 : 20	7 : 10 (.0019)	5 : 12 (.0142)	7 : 13	16 : 3 (.0022)	14 : 5 (.0171)
1 : 19	8 : 9 (.0041)	7 : 10 (.0107)	7 : 13	—	0 : 19 (.0050 +)
2 : 18	10 : 7 (.0021)	8 : 9 (.0147)	8 : 12	17 : 2 (.0015)	15 : 4 (.0152)
3 : 17	11 : 6 (.0025)	9 : 8 (.0171)	8 : 12	0 : 19 (.0020)	1 : 18 (.0121)
4 : 16	12 : 5 (.0025)	10 : 7 (.0179)	9 : 11	17 : 2 (.0038)	16 : 3 (.0123)
5 : 15	13 : 4 (.0023)	11 : 6 (.0174)	9 : 11	0 : 19 (.0008)	2 : 17 (.0193)
6 : 14	14 : 3 (.0018)	12 : 5 (.0159)	10 : 10	18 : 1 (.0022)	17 : 2 (.0089)
6 : 14	—	0 : 17 (.0167)			

TABLE VI

SIGNIFICANT DIFFERENCES IN FOURFOLD CONTINGENCY TABLES— EQUAL SAMPLES; N = 20 AND OVER

N	More A's in Sample (2) than in Sample (1)				Fewer A's in Sample (2) than in Sample (1)			
	Percentage of A's in Sample (1)				Percentage of A's in Sample (1)			
	0	10	25	50	0	10	25	50
	Minimum highly significant percentage differences (P < .005)							
20	35.00	45.00	50.00	45.00	35.00	—	—	45.00
30	26.67	33.33	36.67	36.67	26.67	—	26.67	36.67
40	20.00	27.50	32.50	30.00	20.00	—	22.50	30.00
50	16.00	24.00	28.00	28.00	16.00	—	21.00	28.00
60	13.33	21.67	25.00	25.00	13.33	—	20.00	25.00
70	11.43	18.57	22.86	22.86	11.43	—	17.86.	22.86
80	10.00	17.50	21.25	21.25	10.00	10.00	.17.50	21.25
90	8.89	16.67	20.00	20.00	8.89	10.00	16.11	20.00
100	8.00	15.00	19.00	19.00	8.00	9.00	15.00	19.00
200	4.00	10.00	12.50	13.50	4.00	7.00	11.00	13.50
500	1.60	5.80	7.60	8.40	1.60	4.60	7.00	8.40
1000	0.80	3.90	5.30	5.90	0.80	3.30	5.00	5.90
	Minimum significant percentage differences (P < .025)							
20	25.00	35.00	40.00	35.00	25.00	—	25.00	35.00
30	20.00	26.67	30.00	30.00	20.00	—	23.33	30.00
40	15.00	20.00	25.00	25.00	15.00	—	20.00	25.00
50	12.00	18.00	22.00	22.00	12.00	—	18.00	22.00
60	10.00	16.67	20.00	20.00	10.00	10.00	16.67	20.00
70	8.57	14.29	17.14	18.57	8.57	10.00	14.29	18.57
80	7.50	13.75	16.25	17.50	7.50	8.75	13.75	17.50
90	6.67	12.22	15.56	15.56	6.67	8.89	13.33	15.56
100	6.00	11.00	14.00	15.00	6.00	8.00	12.00	15.00
200	3.00	7.50	9.50	10.50	3.00	6.00	8.50	10.50
500	1.20	4.40	5.80	6.40	1.20	3.60	5.40	6.40
1000	0.60	2.90	4.00	4.50	0.60	2.60	3.80	4.50

Note.—For mode of use see Example 19. N = total number of individuals in each sample. P = Probability. Irregularities in the sequence of percentages are due to the discontinuity of the distributions.

TABLE VII

CHI SQUARE PROBABILITIES

Degrees of freedom	P = 0.10	P = 0.05	P = 0.01
1	2.706	3.841	6.635
2	4.605	5.991	9.210
3	6.251	7.815	11.345
4	7.779	9.488	13.277
5	9.236	11.070	15.086
6	10.645	12.592	16.812
7	12.017	14.067	18.475
8	13.362	15.507	20.090
9	14.684	16.919	21.666
10	15.987	18.307	23.209
11	17.275	19.675	24.725
12	18.549	21.026	26.217
13	19.812	22.362	27.688
14	21.064	23.685	29.141
15	22.307	24.996	30.578
16	23.542	26.296	32.000
17	24.769	27.587	33.409
18	25.989	28.869	34.805
19	27.204	30.144	36.191
20	28.412	31.410	37.566
21	29.615	32.671	38.932
22	30.813	33.924	40.289
23	32.007	35.172	41.638
24	33.196	36.415	42.980
25	34.382	37.652	44.314
26	35.563	38.885	45.642
27	36.741	40.113	46.963
28	37.916	41.337	48.278
29	39.087	42.557	49.588
30	40.256	43.773	50.892

Additional details for one degree of freedom

P	0.99	0.95	0.90	0.70	0.50	0.30
½P	0.495	0.475	0.45	0.35	0.25	0.15
Chi square	0.000157	0.00393	0.0158	0.148	0.455	1.074
P	0.20	0.10	0.05	0.02	0.01	0.001
½P	0.10	0.05	0.025	0.01	0.005	0.0005
Chi square	1.642	2.706	3.841	5.412	6.635	10.827

Note.—Extracted from Table IV of Fisher and Yates's Statistical Tables, with permission of the publishers, Messrs. Oliver and Boyd. For mode of use see Examples 13, 20, and 27. P = probability of obtaining, by random sampling, chi square values as great as, and greater than, the specified value.

TABLE VIII

FOUR-PLACE LOGARITHMS OF FACTORIALS OF NUMBERS UP TO 1000

No.	Log of factorial	No.	Log of factorial	No.	Log of factorial
1	0.0000	51	66.1906	101	159.9743
2	0.3010	52	67.9066	102	161.9829
3	0.7782	53	69.6309	103	163.9958
4	1.3802	54	71.3633	104	166.0128
5	2.0792	55	73.1037	105	168.0340
6	2.8573	56	74.8519	106	170.0593
7	3.7024	57	76.6077	107	172.0887
8	4.6055	58	78.3712	108	174.1221
9	5.5598	59	80.1420	109	176.1595
10	6.5598	60	81.9202	110	178.2009
11	7.6012	61	83.7055	111	180.2462
12	8.6803	62	85.4979	112	182.2955
13	9.7943	63	87.2972	113	184.3485
14	10.9404	64	89.1034	114	186.4054
15	12.1165	65	90.9163	115	188.4661
16	13.3206	66	92.7359	116	190.5306
17	14.5511	67	94.5619	117	192.5988
18	15.8063	68	96.3945	118	194.6707
19	17.0851	69	98.2333	119	196.7462
20	18.3861	70	100.0784	120	198.8254
21	19.7083	71	101.9297	121	200.9082
22	21.0508	72	103.7870	122	202.9945
23	22.4125	73	105.6503	123	205.0844
24	23.7927	74	107.5196	124	207.1779
25	25.1906	75	109.3946	125	209.2748
26	26.6056	76	111.2754	126	211.3751
27	28.0370	77	113.1619	127	213.4790
28	29.4841	78	115.0540	128	215.5862
29	30.9465	79	116.9516	129	217.6967
30	32.4237	80	118.8547	130	219.8107
31	33.9150	81	120.7632	131	221.9280
32	35.4202	82	122.6770	132	224.0485
33	36.9387	83	124.5961	133	226.1724
34	38.4702	84	126.5204	134	228.2995
35	40.0142	85	128.4498	135	230.4298
36	41.5705	86	130.3843	136	232.5634
37	43.1387	87	132.3238	137	234.7001
38	44.7185	88	134.2683	138	236.8400
39	46.3096	89	136.2177	139	238.9830
40	47.9116	90	138.1719	140	241.1291
41	49.5244	91	140.1310	141	243.2783
42	51.1477	92	142.0948	142	245.4306
43	52.7811	93	144.0632	143	247.5860
44	54.4246	94	146.0364	144	249.7443
45	56.0778	95	148.0141	145	251.9057
46	57.7406	96	149.9964	146	254.0700
47	59.4127	97	151.9831	147	256.2374
48	61.0939	98	153.9744	148	258.4076
49	62.7841	99	155.9700	149	260.5808
50	64.4831	100	157.9700	150	262.7569

TABLE VIII—*Continued*

FOUR-PLACE LOGARITHMS OF FACTORIALS OF NUMBERS UP TO 1000—*Continued*

No.	Log of factorial	No.	Log of factorial	No.	Log of factorial
151	264.9359	201	377.2001	251	494.9093
152	267.1177	202	379.5054	252	497.3107
153	269.3024	203	381.8129	253	499.7138
154	271.4899	204	384.1226	254	502.1186
155	273.6803	205	386.4343	255	504.5252
156	275.8734	206	388.7482	256	506.9334
157	278.0693	207	391.0642	257	509.3433
158	280.2679	208	393.3822	258	511.7549
159	282.4693	209	395.7024	259	514.1682
160	284.6735	210	398.0246	260	516.5832
161	286.8803	211	400.3489	261	518.9999
162	289.0898	212	402.6752	262	521.4182
163	291.3020	213	405.0036	263	523.8381
164	293.5168	214	407.3340	264	526.2597
165	295.7343	215	409.6664	265	528.6830
166	297.9544	216	412.0009	266	531.1079
167	300.1771	217	414.3373	267	533.5344
168	302.4024	218	416.6758	268	535.9625
169	304.6303	219	419.0162	269	538.3922
170	306.8608	200	421.3587	270	540.8236
171	309.0938	221	423.7031	271	543.2566
172	311.3293	222	426.0494	272	545.6912
173	313.5674	223	428.3977	273	548.1273
174	315.8079	224	430.7480	274	550.5651
175	318.0509	225	433.1002	275	553.0044
176	320.2965	226	435.4543	276	555.4453
177	322.5444	227	437.8103	277	557.8878
178	324.7948	228	440.1682	278	560.3318
179	327.0477	229	442.5281	279	562.7774
180	329.3030	230	444.8898	280	565.2246
181	331.5607	231	447.2534	281	567.6733
182	333.8207	232	449.6189	282	570.1235
183	336.0832	233	451.9862	283	572.5753
184	338.3480	234	454.3555	284	575.0287
185	340.6152	235	456.7265	285	577.4835
186	342.8847	236	459.0994	286	579.9399
187	345.1565	237	461.4742	287	582.3977
188	347.4307	238	463.8508	288	584.8571
189	349.7071	239	466.2292	289	587.3180
190	351.9859	240	468.6094	290	589.7804
191	354.2669	241	470.9914	291	592.2443
192	356.5502	242	473.3752	292	594.7097
193	358.8358	243	475.7608	293	597.1766
194	361.1236	244	478.1482	294	599.6449
195	363.4136	245	480.5374	295	602.1147
196	365.7059	246	482.9283	296	604.5860
197	368.0003	247	485.3210	297	607.0588
198	370.2970	248	487.7154	298	609.5330
199	372.5959	249	490.1116	299	612.0087
200	374.8969	250	492.5096	300	614.4858

TABLE VIII—*Continued*

FOUR-PLACE LOGARITHMS OF FACTORIALS OF NUMBERS UP TO 1000—*Continued*

No.	Log of factorial	No.	Log of factorial	No.	Log of factorial
301	616.9644	351	742.6373	401	871.4096
302	619.4444	352	745.1838	402	874.0138
303	621.9258	353	747.7316	403	876.6191
304	624.4087	354	750.2806	404	879.2255
305	626.8930	355	752.8308	405	881.8329
306	629.3787	356	755.3823	406	884.4415
307	631.8659	357	757.9349	407	887.0510
308	634.3544	358	760.4888	408	889.6617
309	636.8444	359	763.0439	409	892.2734
310	639.3357	360	765.6002	410	894.8862
311	641.8285	361	768.1577	411	897.5001
312	644.3226	362	770.7164	412	900.1150
313	646.8182	363	773.2764	413	902.7309
314	649.3151	364	775.8375	414	905.3479
315	651.8134	365	778.3997	415	907.9660
316	654.3131	366	780.9632	416	910.5850
317	656.8142	367	783.5279	417	913.2052
318	659.3166	368	786.0937	418	915.8264
319	661.8204	369	788.6608	419	918.4486
320	664.3255	370	791.2290	420	921.0718
321	666.8320	371	793.7983	421	923.6961
322	669.3399	372	796.3689	422	926.3214
323	671.8491	373	798.9406	423	928.9478
324	674.3596	374	801.5135	424	931.5751
325	676.8715	375	804.0875	425	934.2035
326	679.3847	376	806.6627	426	936.8329
327	681.8993	377	809.2390	427	939.4633
328	684.4152	378	811.8165	428	942.0948
329	686.9324	379	814.3952	429	944.7272
330	689.4509	380	816.9749	430	947.3607
331	691.9707	381	819.5559	431	949.9952
332	694.4918	382	822.1379	432	952.6307
333	697.0143	383	824.7211	433	955.2672
334	699.5380	384	827.3055	434	957.9047
335	702.0631	385	829.8909	435	960.5431
336	704.5894	386	832.4775	436	963.1826
337	707.1170	387	835.0652	437	965.8231
338	709.6460	388	837.6540	438	968.4646
339	712.1762	389	840.2440	439	971.1071
340	714.7076	390	842.8351	440	973.7505
341	717.2404	391	845.4272	441	976.3949
342	719.7744	392	848.0205	442	979.0404
343	722.3097	393	850.6149	443	981.6868
344	724.8463	394	853.2104	444	984.3342
345	727.3841	395	855.8070	445	986.9825
346	729.9232	396	858.4047	446	989.6318
347	732.4635	397	861.0035	447	992.2822
348	735.0051	398	863.6034	448	994.9334
349	737.5479	399	866.2044	449	997.5857
350	740.0920	400	868.8064	450	1000.2389

TABLE VIII—*Continued*

No.	Log of factorial	No.	Log of factorial	No.	Log of factorial
451	1002.8931	501	1136.7862	551	1272.8480
452	1005.5482	502	1139.4870	552	1275.5899
453	1008.2043	503	1142.1885	553	1278.3327
454	1010.8614	504	1144.8909	554	1281.0762
455	1013.5194	505	1147.5942	555	1283.8205
456	1016.1783	506	1150.2984	556	1286.5655
457	1018.8383	507	1153.0034	557	1289.3114
458	1021.4991	508	1155.7093	558	1292.0580
459	1024.1609	509	1158.4160	559	1294.8054
460	1026.8237	510	1161.1236	560	1297.5536
461	1029.4874	511	1163.8320	561	1300.3026
462	1032.1520	512	1166.5412	562	1303.0523
463	1034.8176	513	1169.2514	563	1305.8028
464	1037.4841	514	1171.9623	564	1308.5541
465	1040.1516	515	1174.6741	565	1311.3062
466	1042.8200	516	1177.3868	566	1314.0590
467	1045.4893	517	1180.1003	567	1316.8126
468	1048.1595	518	1182.8146	568	1319.5669
469	1050.8307	519	1185.5298	569	1322.3220
470	1053.5028	520	1188.2458	570	1325.0779
471	1056.1758	521	1190.9626	571	1327.8345
472	1058.8498	522	1193.6803	572	1330.5919
473	1061.5246	523	1196.3988	573	1333.3501
474	1064.2004	524	1199.1181	574	1336.1090
475	1066.8771	525	1201.8383	575	1338.8687
476	1069.5547	526	1204.5593	576	1341.6291
477	1072.2332	527	1207.2811	577	1344.3903
478	1074.9127	528	1210.0037	578	1347.1522
479	1077.5930	529	1212.7272	579	1349.9149
480	1080.2742	530	1215.4514	580	1352.6783
481	1082.9564	531	1218.1765	581	1355.4425
482	1085.6394	532	1220.9024	582	1358.2074
483	1088.3234	533	1223.6292	583	1360.9731
484	1091.0082	534	1226.3567	584	1363.7395
485	1093.6940	535	1229.0851	585	1366.5066
486	1096.3806	536	1231.8142	586	1369.2745
487	1099.0681	537	1234.5442	587	1372.0432
488	1101.7565	538	1237.2750	588	1374.8126
489	1104.4458	539	1240.0066	589	1377.5827
490	1107.1360	540	1242.7390	590	1380.3535
491	1109.8271	541	1245.4722	591	1383.1251
492	1112.5191	542	1248.2062	592	1385.8974
493	1115.2119	543	1250.9410	593	1388.6705
494	1117.9057	544	1253.6766	594	1391.4443
495	1120.6003	545	1256.4130	595	1394.2188
496	1123.2958	546	1259.1501	596	1396.9940
497	1125.9921	547	1261.8881	597	1399.7700
498	1128.6893	548	1264.6269	598	1402.5467
499	1131.3874	549	1267.3665	599	1405.3241
500	1134.0864	550	1270.1069	600	1408.1023

TABLE VIII—*Continued*

FOUR-PLACE LOGARITHMS OF FACTORIALS OF NUMBERS UP TO 1000—*Continued*

No.	Log of factorial	No.	Log of factorial	No.	Log of factorial
601	1410.8812	651	1550.7215	701	1692.2299
602	1413.6608	652	1553.5357	702	1695.0762
603	1416.4411	653	1556.3506	703	1697.9232
604	1419.2221	654	1559.1662	704	1700.7708
605	1422.0039	655	1561.9824	705	1703.6190
606	1424.7863	656	1564.7993	706	1706.4678
607	1427.5695	657	1567.6169	707	1709.3172
608	1430.3534	658	1570.4351	708	1712.1672
609	1433.1380	659	1573.2540	709	1715.0179
610	1435.9234	660	1576.0736	710	1717.8691
611	1438.7094	661	1578.8938	711	1720.7210
612	1441.4962	662	1581.7146	712	1723.5735
613	1444.2836	663	1584.5361	713	1726.4266
614	1447.0718	664	1587.3583	714	1729.2803
615	1449.8607	665	1590.1811	715	1732.1346
616	1452.6503	666	1593.0046	716	1734.9895
617	1455.4405	667	1595.8287	717	1737.8450
618	1458.2315	668	1598.6535	718	1740.7011
619	1461.0232	669	1601.4789	719	1743.5578
620	1463.8156	670	1604.3050	720	1746.4152
621	1466.6087	671	1607.1317	721	1749.2731
622	1469.4025	672	1609.9591	722	1752.1316
623	1472.1970	673	1612.7871	723	1754.9908
624	1474.9922	674	1615.6158	724	1757.8505
625	1477.7880	675	1618.4451	725	1760.7109
626	1480.5846	676	1621.2750	726	1763.5718
627	1483.3819	677	1624.1056	727	1766.4333
628	1486.1798	678	1626.9368	728	1769.2955
629	1488.9785	679	1629.7687	729	1772.1582
630	1491.7778	680	1632.6012	730	1775.0215
631	1494.5779	681	1635.4344	731	1777.8854
632	1497.3786	682	1638.2681	732	1780.7499
633	1500.1800	683	1641.1026	733	1783.6150
634	1502.9821	684	1643.9376	734	1786.4807
635	1505.7849	685	1646.7733	735	1789.3470
636	1508.5883	686	1649.6096	736	1792.2139
637	1511.3924	687	1652.4466	737	1795.0814
638	1514.1973	688	1655.2842	738	1797.9494
639	1517.0028	689	1658.1224	739	1800.8181
640	1519.8090	690	1660.9612	740	1803.6873
641	1522.6158	691	1663.8007	741	1806.5571
642	1525.4233	692	1666.6408	742	1809.4275
643	1528.2316	693	1669.4816	743	1812.2985
644	1531.0404	694	1672.3229	744	1815.1701
645	1533.8500	695	1675.1649	745	1818.0423
646	1536.6602	696	1678.0075	746	1820.9150
647	1539.4711	697	1680.8508	747	1823.7883
648	1542.2827	698	1683.6946	748	1826.6622
649	1545.0950	699	1686.5391	749	1829.5367
650	1547.9079	700	1689.3842	750	1832.4118

TABLE VIII—*Continued*

FOUR-PLACE LOGARITHMS OF FACTORIALS OF NUMBERS UP TO 1000—*Continued*

No.	Log of factorial	No.	Log of factorial	No.	Log of factorial
751	1835.2874	801	1979.7907	851	2125.6495
752	1838.1636	802	1982.6949	852	2128.5800
753	1841.0404	803	1985.5996	853	2131.5109
754	1843.9178	804	1988.5049	854	2134.4424
755	1846.7957	805	1991.4107	855	2137.3744
756	1849.6742	806	1994.3170	856	2140.3068
757	1852.5533	807	1997.2239	857	2143.2398
758	1855.4330	808	2000.1313	858	2146.1733
759	1858.3133	809	2003.0392	859	2149.1073
760	1861.1941	810	2005.9477	860	2152.0418
761	1864.0755	811	2008.8567	861	2154.9768
762	1866.9574	812	2011.7663	862	2157.9123
763	1869.8399	813	2014.6764	863	2160.8483
764	1872.7230	814	2017.5870	864	2163.7848
765	1875.6067	815	2020.4982	865	2166.7218
766	1878.4909	816	2023.4099	866	2169.6594
767	1881.3757	817	2026.3221	867	2172.5974
768	1884.2611	818	2029.2348	868	2175.5359
769	1887.1470	819	2032.1481	869	2178.4749
770	1890.0335	820	2035.0619	870	2181.4144
771	1892.9205	821	2037.9763	871	2184.3545
772	1895.8082	822	2040.8911	872	2187.2950
773	1898.6963	823	2043.8065	873	2190.2360
774	1901.5851	824	2046.7225	874	2193.1775
775	1904.4744	825	2049.6389	875	2196.1195
776	1907.3642	826	2052.5559	876	2199.0620
777	1910.2547	827	2055.4734	877	2202.0050
778	1913.1456	828	2058.3914	878	2204.9485
779	1916.0372	829	2061.3100	879	2207.8925
780	1918.9293	830	2064.2291	880	2210.8370
781	1921.8219	831	2067.1487	881	2213.7820
782	1924.7151	832	2070.0688	882	2216.7274
783	1927.6089	833	2072.9894	883	2219.6734
784	1930.5032	834	2075.9106	884	2222.6198
785	1933.3981	835	2078.8323	885	2225.5668
786	1936.2935	836	2081.7545	886	2228.5142
787	1939.1895	837	2084.6772	887	2231.4621
788	1942.0860	838	2087.6005	888	2234.4106
789	1944.9831	839	2090.5242	889	2237.3595
790	1947.8807	840	2093.4485	890	2240.3088
791	1950.7789	841	2096.3733	891	2243.2587
792	1953.6776	842	2099.2986	892	2246.2091
793	1956.5769	843	2102.2244	893	2249.1599
794	1959.4767	844	2105.1508	894	2252.1113
795	1962.3771	845	2108.0776	895	2255.0631
796	1965.2780	846	2111.0050	896	2258.0154
797	1968.1794	847	2113.9329	897	2260.9682
798	1971.0814	848	2116.8613	898	2263.9215
799	1973.9840	849	2119.7902	899	2266.8752
800	1976.8871	850	2122.7196	900	2269.8295

TABLE VIII—*Concluded*

Fᴏᴜʀ-ᴘʟᴀᴄᴇ ʟᴏɢᴀʀɪᴛʜᴍs ᴏғ ғᴀᴄᴛᴏʀɪᴀʟs ᴏғ ɴᴜᴍʙᴇʀs ᴜᴘ ᴛᴏ 1000—*Concluded*

No.	Log of factorial	No.	Log of factorial	No.	Log of factorial
901	2272.7842	936	2376.4993	971	2480.7827
902	2275.7394	937	2379.4711	972	2483.7703
903	2278.6951	938	2382.4433	973	2486.7584
904	2281.6513	939	2385.4159	974	2489.7470
905	2284.6079	940	2388.3891	975	2492.7360
906	2287.5650	941	2391.3627	976	2495.7255
907	2290.5226	942	2394.3367	977	2498.7154
908	2293.4807	943	2397.3112	978	2501.7057
909	2296.4393	944	2400.2862	979	2504.6965
910	2299.3983	945	2403.2616	980	2507.6877
911	2302.3579	946	2406.2375	981	2510.6794
912	2305.3179	947	2409.2139	982	2513.6715
913	2308.2783	948	2412.1907	983	2516.6640
914	2311.2393	949	2415.1679	984	2519.6570
915	2314.2007	950	2418.1457	985	2522.6505
916	2317.1626	951	2421.1238	986	2525.6443
917	2320.1250	952	2424.1025	987	2528.6387
918	2323.0878	953	2427.0816	988	2531.6334
919	2326.0511	954	2430.0611	989	2534.6286
920	2329.0149	955	2433.0411	990	2537.6242
921	2331.9792	956	2436.0216	991	2540.6203
922	2334.9439	957	2439.0025	992	2543.6168
923	2337.9091	958	2441.9839	993	2546.6138
924	2340.8748	959	2444.9657	994	2549.6112
925	2343.8409	960	2447.9479	995	2552.6090
926	2346.8075	961	2450.9307	996	2555.6073
927	2349.7746	962	2453.9138	997	2558.6059
928	2352.7421	963	2456.8975	998	2561.6051
929	2355.7102	964	2459.8815	999	2564.6046
930	2358.6786	965	2462.8661	1000	2567.6046
931	2361.6476	966	2465.8511		
932	2364.6170	967	2468.8365		
933	2367.5869	968	2471.8224		
934	2370.5572	969	2474.8087		
935	2373.5281	970	2477.7954		

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Note.—For Graphs 1–6 (Figs. 6–11) see following inserts.

Fig. 6. Graph I—Confidence limits from Table II—wide limits (P = 0.005). Number of A's in sample: 20 and over. Percentage of A's in sample (represented by curves): 5 to 50. For mode of use see Examples 8 to 10.

– – – – Upper limits.———Lower limits.

TOTAL NUMBER OF INDIVDUALS IN SAMPLE (N)

Fig. 7. Graph 2—Confidence limits from Table II—medium limits (P = 0.025). Number of A's in sample: 20 and over. Percentage of A's in sample (represented by curves): 5 to 50. For mode of use see Examples 8 to 10.
----Upper limits.——Lower limits.

POPULATION PERCENTAGE

TOTAL NUMBER OF INDIVIDUALS IN SAMPLE (N)
(M = 1000)

Fig. 8. Graph 3—Confidence limits from Table II—narrow limits (P = 0.1).
Number of A's in sample: 20 and over. Percentage of A's in sample (represented
by curves): 5 to 50. For mode of use see Examples 8 to 10.
——Upper limits.———Lower limits.

Fig. 9. Graph 4—Confidence limits from Table II—wide limits (P = 0.005). Number of A's in sample: 20 and over. Percentage of A's in sample (represented by curves): 0.1 to 5. For mode of use see Example 12.

– – – Upper limits.———Lower limits.

Fig. 10. Graph 5—Confidence limits from Table II—medium limits (P = 0.025). Number of A's in sample: 20 and over. Percentage of A's in sample (represented by curves): 0.1 to 5. For mode of use see Example 12.
— — — Upper limits.————Lower limits.

Fig. 11. Graph 6—Confidence limits from Table II—narrow limits (P = 0.1). Number of A's in sample: 20 and over. Percentage of A's in sample (represented by curves): 0.1 to 5. For mode of use see Example 12.
———Upper limits.———Lower limits.

THE TREATMENT OF PHOSGENE POISONING WITH TRACHEOTOMY AND SUCTION[1]

By R. A. Waud[2] and Ruth Horner[3]

Abstract

A series of dogs poisoned by exposure to phosgene gas were treated by tracheotomy and suction applied by insertion of a catheter deep into the trachea. By this method of treatment, animals were brought out of a moribund condition and the survival time was definitely increased. The survival rate, however, was essentially the same as that of the control animals. Although digitalis augments the heart beat, clears up irregularities, and keeps the heart beating for a time after respiration has failed, it may be detrimental by increasing the effusion of the fluid into the lung rather than decreasing it. The higher the pregassing concentration of haemoglobin the smaller the amount of fluid that effused into the lungs and the greater was the chance of survival. In practically all animals the point at which haemoconcentration reached a maximum was between 22 and 23 gm. of haemoglobin per 100 mils of blood. On post-mortem examination of dogs dying from phosgene poisoning the stomach was found to be markedly distended with gas and the vessels congested. This adds to the embarrassment of respiration and circulation. The big problem in the treatment of phosgene poisoning is still the finding of some substance or means of limiting or preventing the effusion of fluid into the airways of the lung.

Introduction

After observing a great number of animals dying from phosgene poisoning, one is impressed with the part played by the accumulation of liquid in the airways of the lungs and thus causing the animal to drown in its own fluids. Haemoconcentration occurs regularly in dogs poisoned with phosgene (2). Rabbits are able to keep their blood diluted presumably by withdrawal of fluid from the tissues. However this maintenance of a nearly normal haemoconcentration does not prevent the death of these animals. It is unlikely, therefore, that haemoconcentration is the primary cause of death. In the dog, fluid is lost from the blood and at the same time, it accumulates in the lungs. Therefore, effusion into the lungs should eventually be limited by a certain degree of haemoconcentration.

Because of the paramount part apparently played by the fluids in the lungs in the cause of death, it was decided to direct our treatment toward the removal and possible prevention of the accumulation of this fluid in the lungs. It was, therefore, decided to treat a series of dogs poisoned with phosgene by performing tracheotomy and removing as much fluid as possible by suction.

Methods

Dogs were gassed in a static chamber containing phosgene in a concentration of 0.80 mgm. per liter. Haemoglobin estimations were made imme-

diately before gassing and at regular intervals following. In a preliminary experiment, the dogs were exposed to the gas for a period of 15 min. This exposure, however, was found to be insufficient to kill most of the animals. The time of exposure was, therefore, increased to 20 min. Thirty-two dogs were gassed for the longer period, 17 were treated, and 15 used as controls. After being gassed, the animals were observed continuously day and night until they died or the crisis was over.

After removal from the tank, each animal was kept under observation until signs of pulmonary involvement had developed to the point where the animal was coughing up frothy fluid and in most cases practically in a dying state. Tracheotomy was then performed according to Jackson's technic (1, pp. 382-405) and a tracheal cannula, with two openings to the outside, inserted. If the animal was conscious procaine anaesthesia was used. A No. 18 French rubber catheter attached to a filter pump was then passed into the trachea to the level of bifurcation. A Woulff bottle was connected between the catheter and the filter pump to collect the pulmonary fluid. Sufficient negative pressure was used to keep the fluid flowing through the tube. As lying on its side interferes with the maximum expansion of the animal's chest and proper drainage, the animal was kept in the sitting position. In order to keep as much as possible of the airway clear, the position of the end of the catheter was frequently changed and was kept in the animal until there was little or no fluid coming through. When it became certain that the wet stage had passed, the tracheal cannula was removed but the tracheal opening was not closed.

In a previous report (3), some evidence was presented that suggested that the intravenous administration of theophylline ethylenediamine has a beneficial effect in some cases of phosgene poisoning. Injections of this drug were, therefore, given during the acute wet stage.

Atropine, because of its stimulating effect on the respiratory center, and its effects on vagal innervation of the glands and smooth muscle of the bronchi, was injected into the treated animals. Epinephrine was also injected to dilate the bronchi and decrease the secretions.

As the heart became very rapid and irregular during the stage of acute pulmonary oedema, an attempt was made to control this with digitalis.

Water was given ad libitum but very little was taken by the animals until the wet stage had passed. Oxygen was given as soon as the airway was cleared of fluid.

Results

Often the immediate results have been dramatic. In animals that have become deeply cyanosed and moribund, in a short time after instituting suction, the mucous membranes became pink, the respiration changed from irregular gasps to a deep vigorous type, and the reflexes returned. The flow of fluid in some animals was not continuous but was interrupted by periods of relatively no flow. Later the periods of flow shortened and finally suction

was not necessary. In other animals there seemed to be little decrease in flow over a period of hours. The amount of fluid removed from a lung varied from 100 mils to 450 mils and contained a considerable number of blood cells. The time of occurrence and duration of each gush varied in each animal. During a gush the greater part of the respiratory tree becomes filled with frothy fluid, which if not removed by suction, caused acute asphyxia of the animal. Even after the wet stage is passed, thick tenacious material may plug the trachea and if not removed by suction or by coughing will asphyxiate the animal.
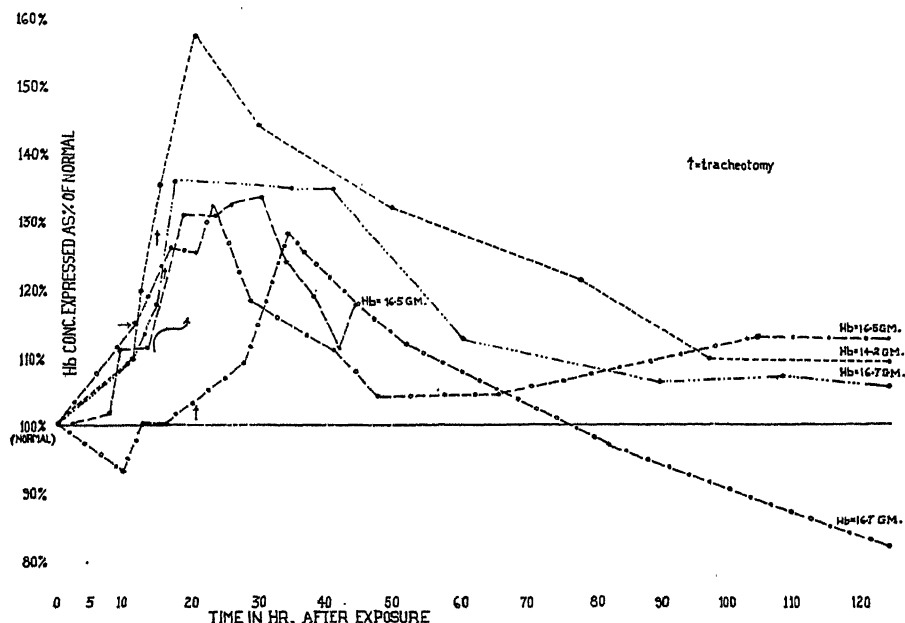
FIG. 1. *Curves showing haemoglobin values of dogs surviving past the wet stage. The time at which suction treatment was started is indicated by arrow.*

The tracheotomy alone was found to facilitate removal of fluid from the trachea and bronchi. It shortened the airway and overcame any obstruction that might be present in the larynx. It also made possible the expulsion of a considerable amount of the frothy fluid and tenacious material by the animal itself, either by coughing or by vomiting-like movements.

Theophylline increased the depth of respiration and in that way improved the condition of the animal for the time being at least. Under the conditions it was used, it would be difficult to determine whether or not it increased the survival time of the animals. No evidence of diminution in pulmonary oedema fluid was noted.

Atropine also caused marked respiratory stimulation and for a time improved the pulmonary ventilation, but there was evidence that a depression followed that could not be overcome by the administration of more atropine. There was no evidence that atropine diminished the pulmonary oedema fluid.

Digitalis increased the force of the heart beat, slowed the rate, and removed the irregularities. In all digitalized dogs that died, respiration failed before the heart.

Following gassing, all the animals showed considerable haemoconcentration. The time at which the maximum concentration was reached varied from 6 to 36 hours, after gassing. The average was 16 hr. The maximum haemoconcentration varied from 126% to 187.5% of normal. The number of grams of haemoglobin contained in 100 mils of blood of each animal before gassing was taken as the normal for that animal. The average normal level for the treated animal that survived was 16.5 gm. of haemoglobin per 100 mils of blood while the average for those animals that were treated and that ultimately died was 15.0 gm. per 100 mils of blood. The average maximum concentration reached in the treated animals that survived was 22.2 gm. In those in which we were able to prolong life but that ultimately died, it was 22.1 gm. of haemoglobin per 100 mils of blood. Graphic representations of the percentage variations of the haemoglobin values together with the time are shown in the graphs (Figs. 1 and 2).
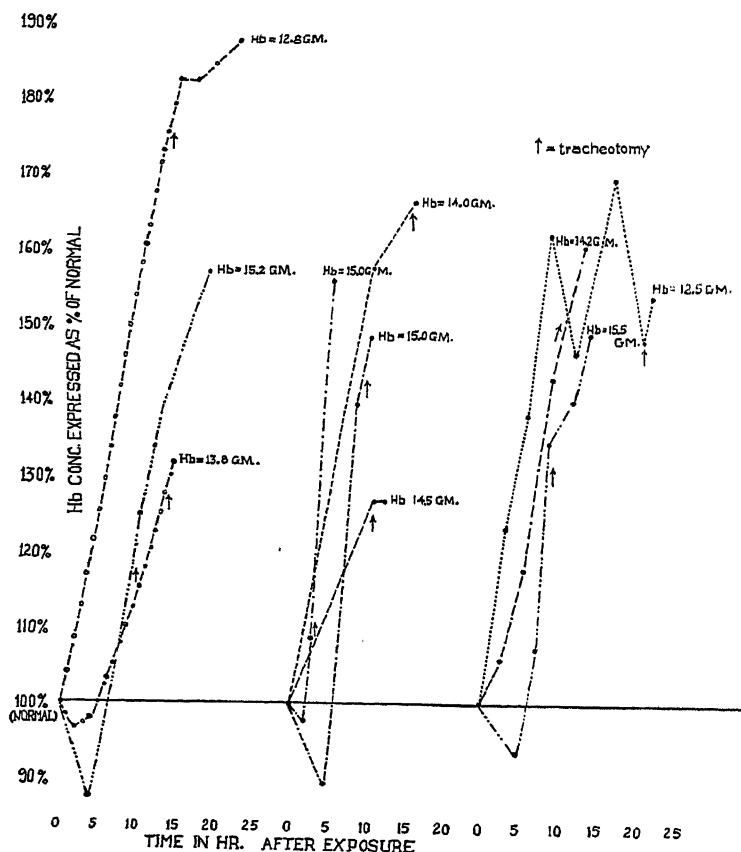
FIG. 2. *Curves showing haemoglobin values of dogs dying during wet stage. The time at which suction treatment was started is indicated by arrow.*

In the 20 min. series 32 dogs were gassed. Fifteen of these were kept as controls and 17 were treated. Two of the treated animals, after being brought out of a moribund condition by tracheotomy, and suction, died later unattended. Of the remaining 15 animals, three were complete survivals. Also three of the control animals survived.

The survival time of the 14 treated animals that died was prolonged in most cases from $\frac{1}{2}$ to 10 hr. One dog survived 116 hr. after tracheotomy and finally died of pneumonia. One lung was consolidated. Another dog survived 27 hr. after tracheotomy but, when apparently in very good condition, died of over-exertion when an attempt was made to administer a capsule containing sulfathiazole. Although this dog was almost moribund, it was not in coma when the tracheotomy was done.

Discussion

In poisoning with phosgene, damage at the alveolar wall results in an effusion of a serum-like material that tends to fill up the alveoli and work into the bronchioles, bronchi, and trachea. Here it is mixed with secretions provoked by the irritating gas.

Variability in the response of individual animals of the same species to an identical concentration and time of exposure to phosgene is now well recognized. In addition to the variations in the physical condition of the animal there are variations in the degree of excitement and activity, the depth, rate, volume, and type of respiration. These factors in turn govern the amount of phosgene entering the lung. To some degree they can be overcome by allowing the animal to remain a sufficient length of time in the tank before adding the gas. The temperature and humidity are also factors. In these experiments, therefore, in addition to the use of control animals, treatment was not instituted until we felt that the animal was in a dying condition. Although this put the treatment at a disadvantage we feel that any prolongation of life was without doubt due to the treatment.

Tracheotomy and suction, temporarily at least, will prevent an animal in an initial acute pulmonary oedema from drowning in its own fluids but not from further accumulation of fluid. In those animals in which the dose is not too large and there is a tendency for the loss of fluid into the airways to cease, it is possible that the treatment may effect a cure.

Neither theophylline ethylenediamine, atropine, nor epinephrine reduced the oedema flow. Isotonic saline solution injected intravenously during the wet stage increased the flow of fluid into the lungs and did not lower the haemoconcentration. After the wet stage had passed, the animals drank considerable water voluntarily. This was not followed by increased moisture in the lungs but by a lowering of the haemoconcentration. Apparently the animal at this stage is able to retain fluids in the blood stream.

There is evidence in these experiments that dogs with a high initial haemo-globin value are more likely to survive. The average initial haemoglobin concentration for those animals that passed the wet stage was 16.5 gm. per 100 mils of blood while the average for those that failed to pass this stage was 14.6 gm. Haemoconcentration in nearly all of the animals ceased when the values reached between 22 and 23 gm. per 100 mils of blood. It would appear that the nearer the initial value is to the maximum, the less fluid will escape into the lungs to embarrass the respiration.

Digitalis controlled the irregularities and rate of the heart and kept it beating for some time after the respiratory center had failed. This was only accomplished after a proper dose was worked out on phosgene poisoned dogs. The total therapeutic dose of digoxin was found to be 0.075 mgm. per kgm. This was given intramuscularly in three divided doses, at four-hour intervals. One-half the quantity was given in the first dose and the remaining half further divided.

Although the animals drown in their own pulmonary fluid, the length of survival or recovery depends, to some degree, on the length of time the heart can maintain adequate circulation in the respiratory center and other vital organs. However, once the respiratory fluid has accumulated to the point where it prevents contact of the outside air with the blood, it becomes impossible for the heart, no matter what its condition, to maintain an adequate partial pressure of oxygen in its own muscle, in the respiratory center and other vital organs.

In dogs dying of phosgene poisoning we were impressed with the amount of distention of the stomach with gas that was found to be present on opening the abdomen. This distension was so great in most animals that movements of the diaphragm were markedly curtailed and the pressure on the heart must have caused it considerable embarrassment. The distension could be caused by air swallowed as a result of vigorous respiratory effect. On the other hand, the origin of the gas may have been the blood stream. In this connection, the vessels on the surface of the stomach were found to be markedly congested and hemorrhagic in practically all animals. In addition, the partial pressure of carbon dioxide in the blood is high because its escape through the lung has been partially cut off. Both of these factors may combine to produce the distension. Tracheotomy was not a factor since the distension occurred in both treated and nontreated animals. The gas has not been analyzed.

The importance of absolute rest and freedom from excitement in recovery from phosgene poisoning cannot be overemphasized. Often a respiratory gush was seen to have been initiated by slight excitement of the animal or a struggle.

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ETUDE DES PROPRIETES PHARMACOLOGIQUES DE L'ANNOTININE ET DE LA LYCOPODINE[1]

Par Guy Marier[2] et Richard Bernard[3]

Sommaire

Les auteurs du présent travail ont étudié l'annotinine et la lycopodine, alcaloïdes extraits respectivement de *Lycopodium annotinum* et de *Lycopodium clavatum*, au point de vue pharmacologique. La dose léthale moyenne de ces deux alcaloïdes, calculée à la suite d'injections intrapéritonéales chez les souris, est de 166.2 mg./kg. pour l'annotinine et de 78.41 ± 1.06 mg./kg. pour la lycopodine. Ces alcaloïdes provoquent, à doses toxiques, des convulsions toniques et cloniques, de l'asphyxie et de la paralysie chez la grenouille, la souris et le lapin. Cette paralysie, dans le cas de l'annotinine, semble affecter surtout les membres postérieurs. A doses sublethales, ces alcaloïdes provoquent l'augmentation de l'amplitude des mouvements respiratoires et la diminution de fréquence de ces mouvements. Chez le lapin, l'annotinine provoque une hypertension, une vasodilatation périphérique, un myosis prononcé de l'oeil, une hyperglycémie importante causée par une décharge d'adrénaline, une hypothermie qui affecte non seulement la température normale, mais encore la fièvre, et une inversion de l'accident T sur l'électrocardiogramme. L'annotinine et la lycopodine ont une action inotrope positive sur le coeur de grenouille et une action inotrope négative sur le coeur de lapin. L'annotinine provoque la diminution de l'amplitude de la contraction intestinale et l'augmentation de celle de l'utérus. La lycopodine, au contraire, augmente l'amplitude de la contraction intestinale et utérine. Ce même alcaloïde cause une hypothermie plus faible et de moindre durée que celle causée par l'annotinine. Il n'a aucune influence sur le glycémie et ne provoque pas de myosis. L'annotinine et la lycopodine n'ont aucune influence sur les globules sanguins et n'ont aucun pouvoir antibiotique.

Introduction

Les Lycopodes sont des Ptéridophytes de la famille des Lycopodiacées. Ce groupe, largement répandu dans la Province de Québec, renferme un grand nombre d'alcaloïdes qui, pour la plupart, ont été étudiés au point de vue chimique par Manske et Marion (10-17), du Conseil National des Recherches du Canada. Ces deux auteurs ont isolé, à partir des différentes espèces de Lycopodes, plus d'une trentaine d'alcaloïdes nouveaux, dont l'annotinine. Ils ont trouvé, de plus, quelques alcaloïdes connus, dont la lycopodine.

On connaît peu de choses sur la structure chimique de ces deux alcaloïdes que sont l'annotinine et la lycopodine, Manske et Marion (11) donnent à l'annotinine la formule brute suivante: $C_{16}H_{21}O_3N$ avec point de fusion à 232° C. La lycopodine isolée pour la première fois par Bödeker en 1881 (3), puis de nouveau par Achmatowicz (1) en 1938 et Manske et Marion (10) en 1943, a la formule brute suivante: $C_{16}H_{25}ON$ avec point de fusion à 115–116° C. Manske et Marion (14) ont émis l'hypothèse que la lycopodine possède un noyau quinoléique hydrogéné et que l'oxygène y est présent sous forme d'ester cyclique.

Au point de vue pharmacologique, les travaux d'importance qui ont été publiés sur le sujet sont ceux d'Oficjalski (19) sur la toxicité des alcaloïdes de Lycopodes (préparations galéniques), de Nikinorow (18) sur les propriétés antipyrétiques de ces alcaloïdes (préparations galéniques), d'Achmatowicz (1) sur la lycopodine, laquelle cause une paralysie du système nerveux central et périphérique chez la grenouille, ainsi qu'une action tonique sur la respiration. Finalement, en 1945, Lee et Chen (9) rapportent que l'annotinine et la lyco-podine, à doses appropriées, causent de l'incoordination musculaire et de la paralysie chez la grenouille. A faibles doses, ces deux alcaloïdes font aug-menter la pression artérielle du chat. Toujours, d'après Lee et Chen, la lycopodine provoque la contraction de l'intestin et de l'utérus isolés.

Partie experimentale

Extraction

L'extraction de l'annotinine a été faite à partir de *Lycopodium annotinum* selon la méthode décrite par Manske et Marion (10 et 11). L'annotinine, telle qu'extraite a un point de fusion à 232° C., a été transformée pour fin d'injection en chlorhydrate d'annotinine avec point de fusion à 285° C. La lycopodine a été extraite de *Lycopodium clavatum* selon une méthode basée sur celle de Marion (10 et 11) pour les alcaloïdes totaux et sur celle d'Achmatowicz (1) pour la séparation et la purification de la lycopodine; extrait ainsi, l'alca-loïde a son point de fusion à 115–116° C. Le point de fusion du chlorhydrate de lycopodine est à 358° C.

Toxicité

Nous avons procédé à la détermination de la dose léthale moyenne (D.L. 50) à la suite d'injection intrapéritonéales chez la souris. Cette détermination pour l'annotinine a été faite d'après la méthode de Behrens (4) et a donné la valeur suivante: 166.2 mg./kg. La dose léthale moyenne de la lycopodine, calculée d'après la méthode de Karber (4) a atteint 78.41 ± 1.06 mg./kg.

Au cours de l'injection de doses toxiques de l'un ou de l'autre alcaloïde, nous avons observé des réactions semblables chez la grenouille: l'annotinine (à la dose de 300 à 450 mg./kg.) et la lycopodine (à la dose de 50 à 200 mg./kg.), injectées dans le sac lymphatique dorsal provoquent de l'incoordination muscu-laire évidente surtout aux pattes postérieures, et de la paralysie assez com-plète, allant souvent jusqu'à faire croire à la mort de l'animal. Chez la souris, l'injection intrapéritonéale d'annotinine (150–250 mg./kg.) et de lycopodine (50–160 mg./kg.) provoque l'hyperexcitabilité, de convulsions toniques et cloniques, de l'asphyxie et de la paralysie. La paralysie provoquée par l'annotinine semble se localiser aux pattes postérieures. Chez le lapin: l'injection intraveineuse de lycopodine (70–200 mg./kg.) et d'annotinine (150–250 mg./kg.) provoque de l'hyperexcitabilité, des convulsions et de l'asphyxie. L'annotinine provoque de plus une paralysie des membres postérieurs et un myosis de la pupille. La lycopodine provoque la défécation et une vasodilatation périphérique beaucoup plus prononcée que celle causée par l'injection d'annotinine.

Circulation

Pression artérielle et respiration

L'annotinine en injection intraveineuse (30–100 mg./kg.) provoque chez le lapin anesthésié à l'uréthane, immédiatement après l'injection, une hypotension très rapide et de courte durée, puis une hypertension qui se prolonge pendant un temps indéterminé. Cette hypertension varie de 30 à 53 millimètres de mercure. La vagotomie et l'injection subséquente de l'alcaloïde n'amènent pas de résultats différents.

Chez le chat et chez le chien, l'annotinine, aux mêmes doses, provoque une chute prononcée de la pression artérielle, puis une élévation graduelle de la pression jusqu'à un niveau encore en-dessous de la pression normale. Cette hypotension se maintient pendant un temps indéterminé.

La lycopodine injectée par voie intraveineuse à la dose de 30 mg./kg. cause tant chez le lapin que chez le chat une hypotension maxima immédiatement après l'injection. Par la suite, la pression s'élève un peu, tout en demeurant plus basse que la pression normale, et ce pendant un temps indéterminé.

L'effet de l'annotinine et de la lycopodine semble additif et proportionnel à la dose donnée.

La respiration est quelque peu modifiée par l'annotinine et la lycopodine. La fréquence respiratoire diminue et l'amplitude augmente.

Coeur isolé et perfusé

Les perfusions sont faites selon la méthode de Straub (21) pour les coeurs d'animaux à sang froid et celle de Langendorff (7) pour les coeurs d'animaux à sang chaud.

Les coeurs de grenouilles sont perfusés avec des solutions de lycopodine à 1 : 200,000. Au début, la lycopodine a un effet inotrope positif. Par la suite, l'action inotrope a tendance à devenir négative. Sur les coeurs isolés de lapins, la lycopodine (1 : 50,000 à 1 : 400,000) a une action inotrope négative prononcée. Dans les deux cas, la lycopodine ne semble pas affecter sensiblement la fréquence des contractions.

L'annotinine (3 : 50,000 à 3 : 200,000) a sur le coeur de grenouille une action inotrope positive avec une tendance au chronotropisme négatif. Sur les coeurs de lapins, cet alcaloïde (1 : 25,000 à 1 : 200,000) a une action inotrope négative avec tendance au chronotropisme négatif.

On voit donc que les deux alcaloïdes ont, sur le coeur de grenouille d'une part, et sur le coeur de lapin d'autre part, sensiblement la même action.

Electrocardiogramme

La lycopodine, même à des doses de 30 mg./kg., n'affecte pas l'électrocardiogramme du lapin. Par contre, l'annotinine, à la dose de 30–75 mg./kg. chez le lapin et de 50–100 mg./kg. chez le chat, amène la disparition ou l'inversion de l'accident T. Ce changement se produit, tant chez l'animal normal que chez l'animal anesthésié. Il est, de plus, réversible, l'accident T redevenant normal après quelques heures, ceci prouve que si le myocarde est affecté, il n'est pas endommagé.

Muscles lisses

Les enregistrements des contractions intestinales et utérines sont faits selon la méthode de Magnus (8).

Intestin isolé

L'annotinine à la concentration de 1 : 6000 à.1 : 25,000 a été étudiée sur des intestins isolés de rats, cobayes, lapins et chats.   A ces concentrations, l'alcaloïde diminue l'amplitude des contractions sans en diminuer la fréquence. De plus, l'intestin de lapin relâche son tonus.   A des doses plus fortes, tous les mouvements automatiques sont inhibés par l'annotinine.

La lycopodine a un effet contraire à celui de l'annotinine sur les intestins de rats et de cobayes.   A des concentrations de l'ordre de 1 : 12,500 à 1 : 50,000, la lycopodine augmente l'amplitude des mouvements pendulaires de l'intestin sans en changer la fréquence.

Utérus isolé

L'annotinine et la lycopodine provoquent la contraction utérine.   Si la contraction existe déjà, les deux alcaloïdes l'amplifient.   La concentration d'annotinine employée est de 1 : 5000 à 1 : 12,500 pour les utérus de rats, de cobayes et de lapins.   Celle de lycopodine est de 1 : 16,500 pour les utérus de cobayes.

La réaction de l'intestin et de l'utérus, à l'annotinine et à la lycopodine, laisse supposer que le premier alcaloïde peut être considéré comme un excitant sympathique et le second comme un excitant parasympathique.

Température corporelle

La méthode suivie pour déceler l'action de l'annotinine et de la lycopodine sur la température corporelle est celle dérivée de l'essai pour les substances pyrogènes (20).   Les substances employées sont passées au filtre Seitz pour les débarrasser de toutes substances pyrogènes.   Pour l'annotinine, 10 lapins servent à l'expérience, dont cinq comme témoins ne recevant que du chlorure de sodium 9 : 1000.   Les cinq autres reçoivent en injection intraveineuse de l'annotinine à la dose de 50 mg./kg.   Pour la lycopodine, 8 lapins sont employés à l'expérimentation, dont 4 comme témoins ne recevant que du chlorure de sodium à 9 : 1000; les 4 autres reçoivent par voie intraveineuse une dose de lycopodine de 30 mg./kg.

L'annotinine et la lycopodine font baisser la température corporelle.   La chute due à l'annotinine est rapide et sensiblement plus prolongée que celle due à la lycopodine.   La chute réelle de température, une heure et quart après l'injection d'annotinine est de l'ordre de 1.30°F., tandis que celle due à la lycopodine une heure après l'injection est de l'ordre de 0.78° F.

L'annotinine agit non seulement sur la température normale mais encore sur la fièvre provoquée par l'injection intramusculaire de lait bouilli.   Dans ce cas, l'hypothermie moyenne provoquée est, après une heure, de l'ordre de 3.52° F., l'ordre de grandeur de la fièvre étant de 0.96° F. et la chute moyenne de température étant de 2.56° F. (Fig. 1).

Après quatre ou cinq heures, la température est redevenue normale tant chez les fébricitants que chez les individus normaux ayant reçu de l'annotinine. L'annotinine est donc un antithermisant et un fébrifuge. La lycopodine est aussi, mais à une moindre degré, un antithermisant et probablement un fébrifuge.
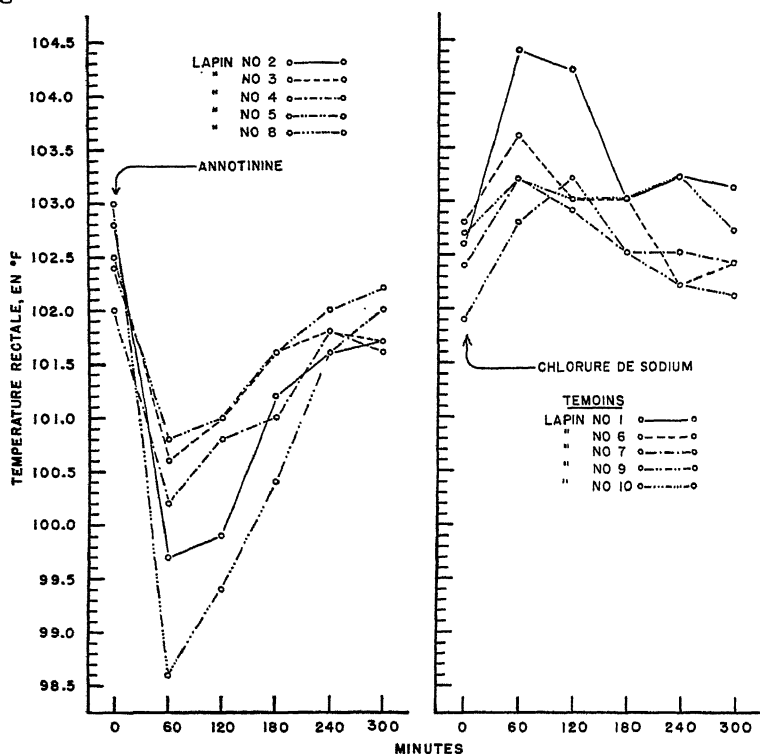
Fig. 1. *Annotinine et fièvre.*

La lycopodine semble avoir cette propriété d'agir sur la température, non pas par action directe sur le centre thermorégulateur, mais bien par la vaso-dilatation périphérique qu'elle produit. Le centre thermorégulateur, et c'est ce pourquoi la température remonte si vite, entre en action pour corriger l'abaissement de la température provoquée par la déperdition de chaleur. L'annotinine agit sur la température en l'abaissant, non seulement parce qu'elle provoque une vasodilatation mais encore parce qu'elle agit sur le centre thermorégulateur qui est déprimé. C'est ce qui explique que la température prenne autant de temps à redevenir normale.

Pouvoir antibiotique

L'annotinine et la lycopodine, même à la concentration de 1%, n'ont aucun pouvoir antibiotique sur des cultures de Staphylocoques dorés (*Staphylococcus aureus*), que ce soit sur bouillon nutritif ou sur gélose nutritive.

Numération globulaire

L'annotinine injectée par voie intraveineuse, à la dose de 50 mg./kg., n'a aucun effet sur le nombre des globules rouges du sang. Le nombre des globules blancs augmente cependant légèrement mais on ne peut dire si cette augmentation est due à l'annotinine ou aux substances pyrogènes qu'aurait pu contenir la solution (2).

Glucose sanguin

La lycopodine à la dose de 30 mg./kg. en injection intraveineuse ne présente aucun effet sur la glycémie. Par contre, l'annotinine s'avère un hyperglycémiant très puissant. La méthode suivie pour l'étude de l'action de l'annotinine sur la glycémie est la microméthode de Folin (6), méthode que l'on a adaptée à l'électrophotomètre de Fisher (5). L'annotinine est injectée par voie intraveineuse à raison de 50 mg./kg. Immédiatement près l'injection, les lapins recevant de l'annotinine subissent une augmentation de glucose sanguin. Cette augmentation est maxima après trois heures et dure de sept à huit heures. L'augmentation moyenne maxima de glucose est de 102.7 mg. par 100 cc. de sang (Fig. 2).

L'augmentation de glucose peut être due à l'action directe de l'annotinine sur la fonction glycogénique. Elle peut se faire aussi par l'intermédiaire de certaines glandes dont la sécrétion serait activée par l'annotinine. Ainsi, l'annotinine peut exciter les glandes surrénales et provoquer une décharge d'adrénaline dans l'organisme, laquelle décharge est responsable de l'hyperglycémie.

Pour ce prouver, nous avons surrénalectomisé totalement un groupe de lapins et les avons maintenus en survie au moyen d'une injection quotidienne d'un demi-centimètre cube d'extrait de glande surrénale (Adrenal Cortical Extract, don des laboratoires Connaught). L'injection subséquente d'annotinine à ces lapins n'amène plus d'hyperglycémie, mais bien une hypoglycémie peu prononcée quoique significative.

L'annotinine produit donc une forte hyperglycémie provenant, non par action directe de l'alcaloïde sur la fonction glycogénique, mais indirectement par l'intermédiaire de l'adrénaline. L'annotinine, en faisant abstraction de l'adrénaline, est un hypoglycémiant léger.

Pupille

L'annotinine et la lycopodine ne réagissent pas de façon semblable sur la pupille de l'oeil. La lycopodine n'a aucun effet sur la pupille que ce soit à la suite d'instillation, d'injection intraveineuse ou sous-cutanée. Au contraire, l'injection intraveineuse d'annotinine (50–100 mg./kg.) produit un myosis très prononcé qui se fait sentir presqu'immédiatement après l'injection et qui dure de deux à trois heures. Après une heure et demie, le myosis est maximum, la pupille ayant diminué de plus de moitié. L'injection sous-cutanée produit la même réaction, mais celle-ci est beaucoup plus lente à apparaître.

L'instillation dans le cul-de-sac de l'oeil d'une solution d'atropine à 1% ou l'injection intraveineuse d'atropine produisent par paralysie des terminaisons

parasympathiques de l'oculaire commun, une mydriase intense qui, cependant, ne peut résister à l'injection d'annotinine; la mydriase due à l'atropine disparaît aussitôt pour faire place à un myosis prononcé.
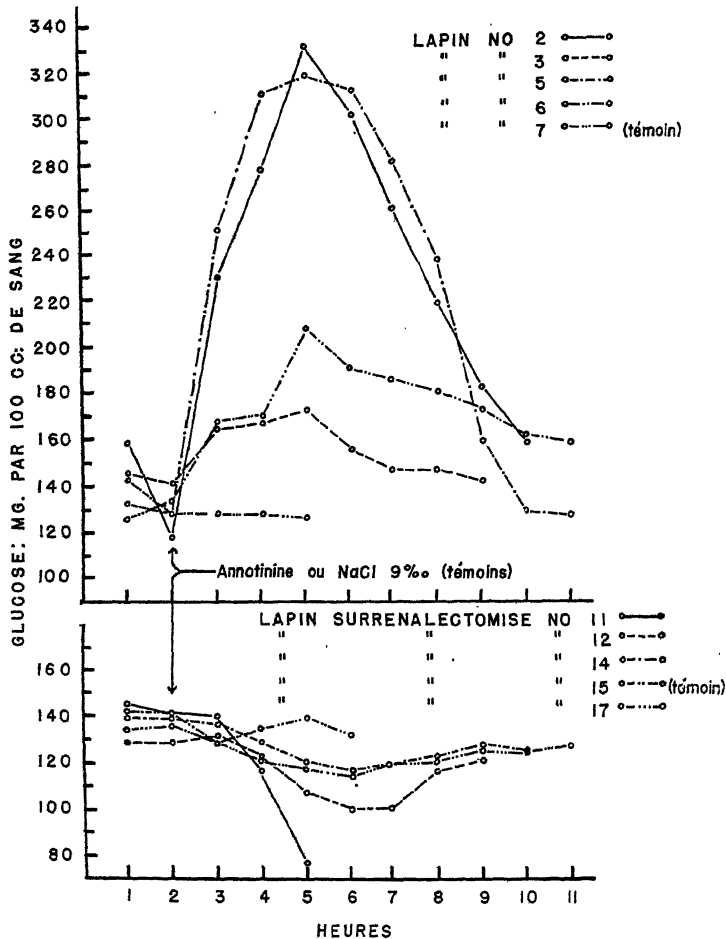
FIG. 2. *Annotinine et glucose sanguin.*

L'instillation dans l'oeil d'une solution d'annotinine à 4% produit un myosis léger et à peine perceptible. Toutefois, l'extirpation des deux yeux d'un lapin et l'immersion de l'un dans une solution à 1 pour mille, nous montrent mieux ce myosis: l'oeil plongé dans la solution à 4% a la pupille beaucoup plus contractée que l'autre.

Nous croyons donc que l'annotinine produit ce myosis de la pupille par action locale sur le muscle radiaire de l'iris. Ce myosis est le fait d'un excitant parasympathique.

Discussion

L'annotinine, bien que moins toxique que la lycopodine, se montre plus active, du point de vue pharmacologique que cette dernière. L'action de la lycopodine sur les principales fonctions de l'organisme et surtout son action sur les muscles lisses, en font un excitant parasympathique. On ne peut dire toutefois la même chose de l'annotinine. Certains effets de cet alcaloïde, comme son action sur les muscles lisses de l'intestin et de l'utérus, son effet sur la pression artérielle de lapin et même son action sur le glycémie, en font un excitant sympathique. Par contre, son action sur le glucose sanguin du lapin surrénalectomisé et surtout son action sur la pupille, en font un excitant parasympathique. On semble donc en présence d'un excitant mixte agissant dans un sens sur certains organes, et dans un autre sens sur d'autres organes.

L'action de la lycopodine et de l'annotinine sur le système nerveux central est de moindre importance si on la compare à celle qu'ont ces deux drogues sur le système nerveux autonome.

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EXPERIENCES WITH THE 'VENUS' HEART METHOD FOR DETERMINING ACETYLCHOLINE[1]

By DONALD B. TOWER[2] AND DONALD McEACHERN[3]

Abstract

Experiences with a little known method for determining acetylcholine in biological materials are reported. The test object is the isolated ventricle of the quahaug, *Venus mercenaria*, a preparation that is stable for many hours. Its minimal sensitivity for acetylcholine is between 0.005 and 0.01 μgm. %. The method is simple and highly specific, and the preparation may be used repeatedly, as often as every 5 to 10 min. Details of the method with illustrative results are given.

Introduction

The detailed study of inter- and intracellular metabolism demands precise micro methods for the analysis of cell enzymes, substrates, and metabolites. This is particularly true for a substance such as acetylcholine that is active in very minute quantities. Present methods for the determination of acetylcholine are not satisfactory because of (a) their lack of sensitivity to micro amounts of acetylcholine, (b) the instability of the acetylcholine molecule, and (c) the chemical and pharmacological similarity of acetylcholine to other constituents of biological materials. Chemical analysis will not detect less than about 0.1 mgm. of pure substance. The inherent difficulties in the use of established biological methods are well known and will not be discussed.

We wish to report our experiences with a little known method of high sensitivity and specificity, employing the isolated ventricle of the mollusc, *Venus mercenaria*. This is the familiar salt water quahaug. The sensitivity of the 'Venus' heart to acetylcholine was originally reported by Prosser and Prosser (7) in 1937 and has been further studied by Smith and Levin (9), Jullien *et al.* (4), Smith and Glick (8), and Prosser (6). Adaptation of the method for biological assay of acetylcholine was described in 1943 by Wait (10) and subsequently by Welsh (11). Comparison with other biological methods of assay for acetylcholine demonstrates the high sensitivity of the isolated 'Venus' ventricle (see Table I) (1; 3, p. 327; 10).

Method

The fresh molluscs may be obtained inexpensively from various biological supply houses.* They are easily stored by simple dry refrigeration at about 5° C. and remain in satisfactory condition for at least two weeks. The test

TABLE I

SENSITIVITIES OF SOME BIOLOGICAL METHODS FOR THE DETERMINATION OF
ACETYLCHOLINE

Preparation	Minimal amount detectable, in μgm. %* (1 ; 3, p. 327; 10)
Frog rectus abdominis (eserinized)	2.0
Frog heart (Straub's method)	1.0
Dorsal leech muscle (eserinized)	0.2
Isolated ventricle of *Venus mercenaria*	0.005 – 0.01

* μgm. % = *micrograms per 100 milliliters.*

preparation can be set up with ease and rapidity. The hinge overlying the heart is located and both shells are cracked to allow uncapping of the pericardial sac (Fig. 1). The sac is entered superficially to expose the ventricle. Ligatures are placed at the auriculoventricular junction, and the ventricle is isolated by cutting it free from auricular and intestinal attachments. In the accompanying photograph (Fig. 1) the ventricle has been injected with a dye (Kiton fast green V) to allow better demonstration. Some dye has passed out along the intestinal vessels to outline the intestine. Ordinarily, however, the ventricle is seen as an almost colorless transparent organ. The ventricle is then mounted in a constant temperature chamber (of 10 to 20 ml. volume) by means of the ligatures previously tied in place (Fig. 2). Gentle rapid handling at this stage is the secret of obtaining a satisfactory preparation.

The ventricle is suspended in sea water. Where sea water is not readily available an artificial medium may be used, based on the analysis of the *Venus mercenaria* blood by Cole (2)†. The latter has proved satisfactory in our hands. A constant temperature bath, operating in the range of 10° to 15° C., provides added stability to the preparation, since the sensitivity of the organ varies slightly with temperature (10). Ventricular contractions

* *We obtained shipments of several dozen fresh Venus mercenaria every two weeks from the Marine Biological Laboratories, Woods Hole, Mass.*

† *Artificial sea water formula modified from Lyman and Fleming (5), Wait (10), and Welsh (12):*

$NaCl$	30 gm./liter		$NaHCO_3$		0.2 gm./liter
$MgSO_4 . 7H_2O$	5.1 "		Glucose		0.25 "
$CaCl_2 . H_2O$	1.1 "		$NaH_2PO_4 . H_2O$	0.5M 1.0	ml./liter
KCl	0.9 "		$Na_2CO_3 . H_2O$	0.5M 0.5	"

$$pH = 7.0 \ to \ 7.4$$

PLATE I

TOP VIEW

INTESTINE

TIE ON
  A-V JUNCTION

VENTRICLE
  IN SITU

LATERAL VIEW

POSITION
  OF HEART

UNCAPPING
  PORTION
  OF SHELL
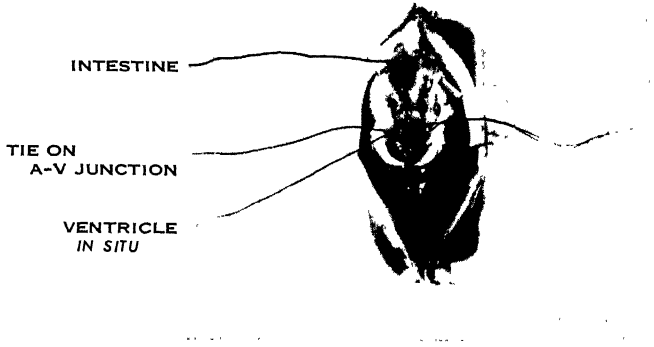
SITE OF
  HAMMER
  BLOW
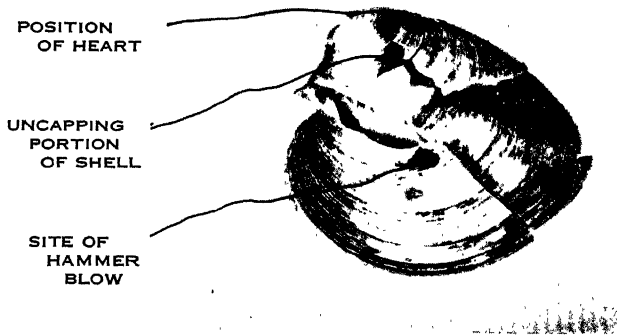
FIG. 1.   *Venus mercenaria.*   *Technique for isolation of ventricle.*

PLATE II
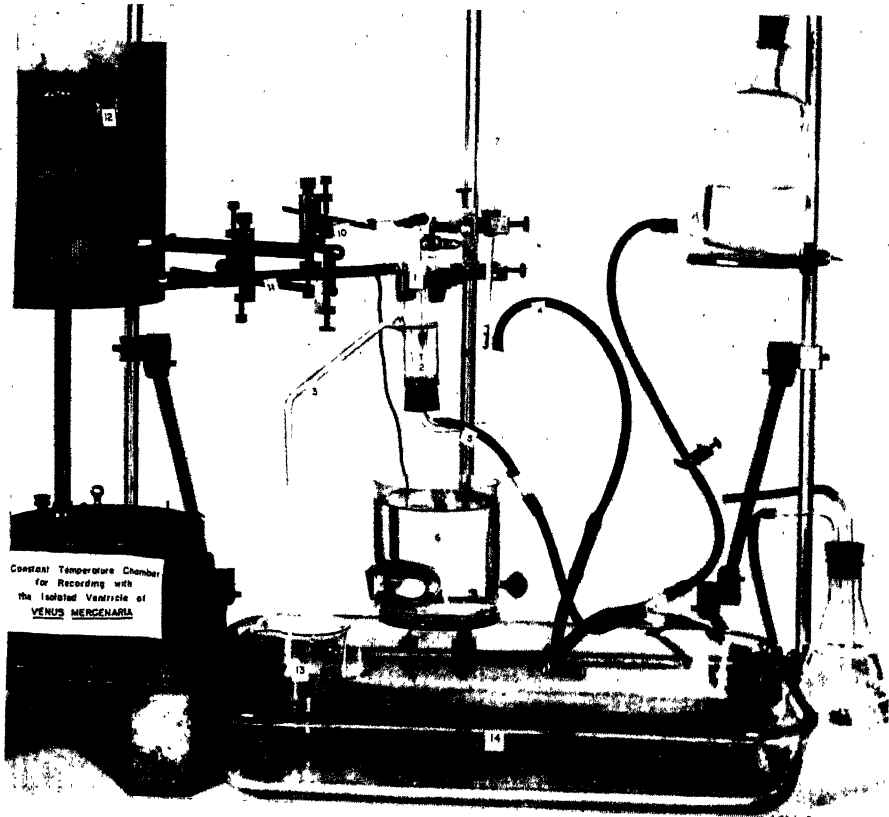
FIG. 2. *1. = Constant temperature bath. 2. = Suspended ventricle. 3. = Chamber overflow. 4. = Sea water inflow. 5. = Air inflow. 6. = Constant temperature bath (lowered to permit visualization of chamber). 7. = Thermometer. 8. = Sea water reservoir. 9. = Air inflow trap bottle. 10. = Light heart lever. 11. = Signal lever. 12. = Kymograph drum. 13. = Overflow graduated beaker. 14. = Overflow dish.*

PLATE III
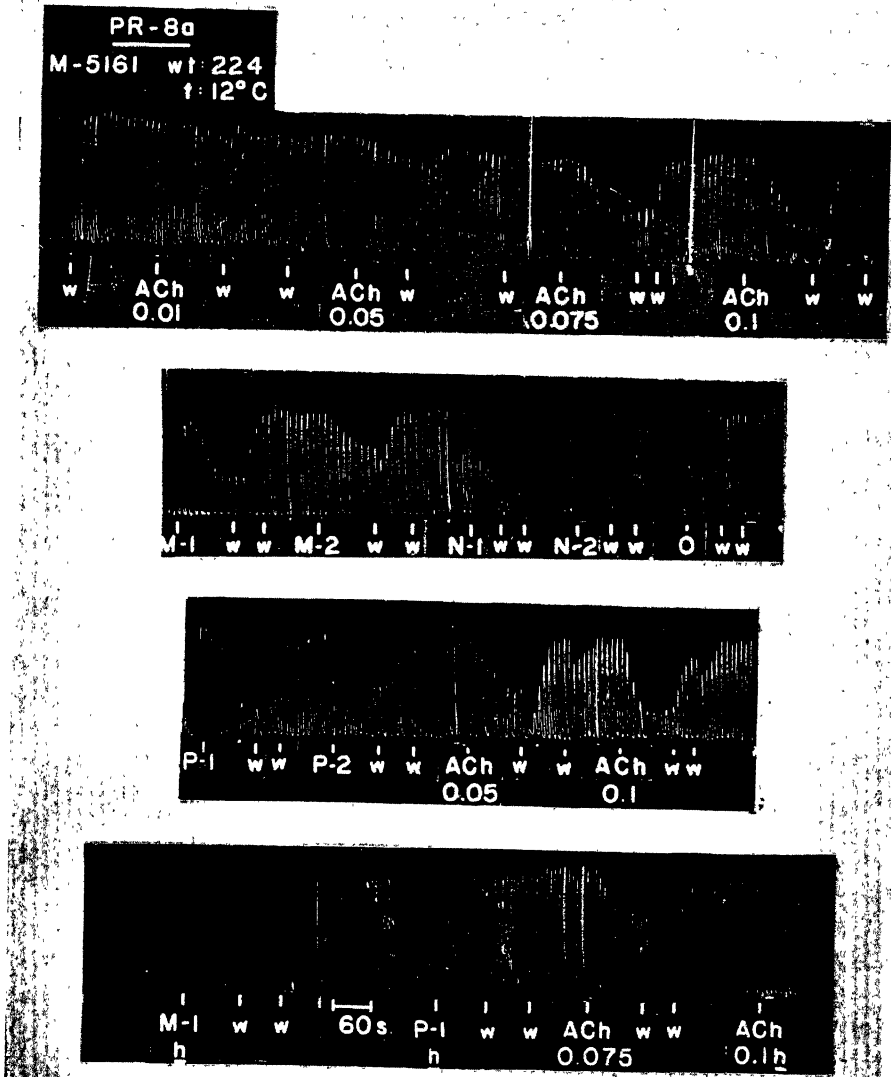
FIG. 3. *Kymograph tracing of acetylcholine assay using the isolated ventricle of Venus mercenaria.*
Ach = *Acetylcholine in μgm. %; w* = *Wash; h* = *Hydrolyzed samples; M, N, O, P* = *Samples for testing.*

usually begin immediately and are recorded upon a smoked kymograph drum using a light heart lever counterweighted to 250 mgm. Gentle tension on the recording lever or several washings with sea water will stimulate recalcitrant ventricles to begin contracting. Aeration, although not necessary for functioning of the organ, is employed. Air is bubbled constantly through a small needle and keeps the bathing solution well mixed. The sea water medium is changed by overflow washing to avoid drying the ventricle (Fig. 2). The preparation reaches a steady state and is ready for testing 30 to 45 min. after cracking the shell.

Results

, The effect of acetylcholine upon ventricular contractions is one of partial or complete inhibition of systolic contraction without significant effect upon the rate (Fig. 3). The extent of inhibition is dependent upon the amount of acetylcholine present. The threshold of the preparation is determined with a set of fresh aqueous solutions of known concentration. The threshold is usually about 0.01 μgm. %. (Occasionally it may be as low as 0.00025 μgm. %.) Unknown solutions may then be compared. Such a record is illustrated in Fig. 3. Two or three generous washings and an interval of 5 to 10 min. are sufficient to prepare the ventricle for the next test sample. Unknown samples are bracketed by tests on known solutions in the usual manner. The preparation is stable over many hours and gives a more satisfactory record than the frog or leech muscle.

Since the inhibition of ventricular contraction caused by various concentrations of acetylcholine is quantitative, a graph plotting percentage inhibition of contraction against acetylcholine concentration may be constructed. The graph shown in Fig. 4 is plotted from the record illustrated in Fig. 3. The curve is sigmoid with a linear central portion approximately as indicated (12). Repeated duplication of results may be obtained.

The isolated ventricle of *Venus mercenaria* is highly specific (12). It is insensitive to most other constituents of biological tissues and fluids, in particular potassium, histamine, and adrenaline. It is not susceptible to pH changes over a wide range (at least between pH 5 and 8.5). It is also insensitive to anticholinesterases such as physostigmine (eserine), prostigmine, and di-isopropylfluorophosphate (DFP) (4, 12). In active biological samples the identity of acetylcholine can be established through its destruction upon subjection to alkali-heat hydrolysis (as shown in Fig. 3).

Like many biological preparations the 'Venus' ventricle shows seasonal variations in its responsiveness to acetylcholine. This is particularly noticeable in summer months. Dr. Welsh has brought to our attention the fact that poor preparations can be restored to normal by the addition of ergot alkaloids to the sea water medium to give a final concentration of 1 : 1,000,000 (12). We have found ergometrine (ergonovine) the most satisfactory ergot derivative for this purpose. This addition will also make a good preparation useful again 24 hr. or more after being first set up. Ordinarily such a ventricle,

although still contracting after 24 hr., would have lost stability and sensitivity to acetylcholine.

Our use of the method has been confined to known solutions and to samples of human and animal cerebrospinal fluid and blood serum. We have found the simplicity and superiority of this method over previous methods outstanding.
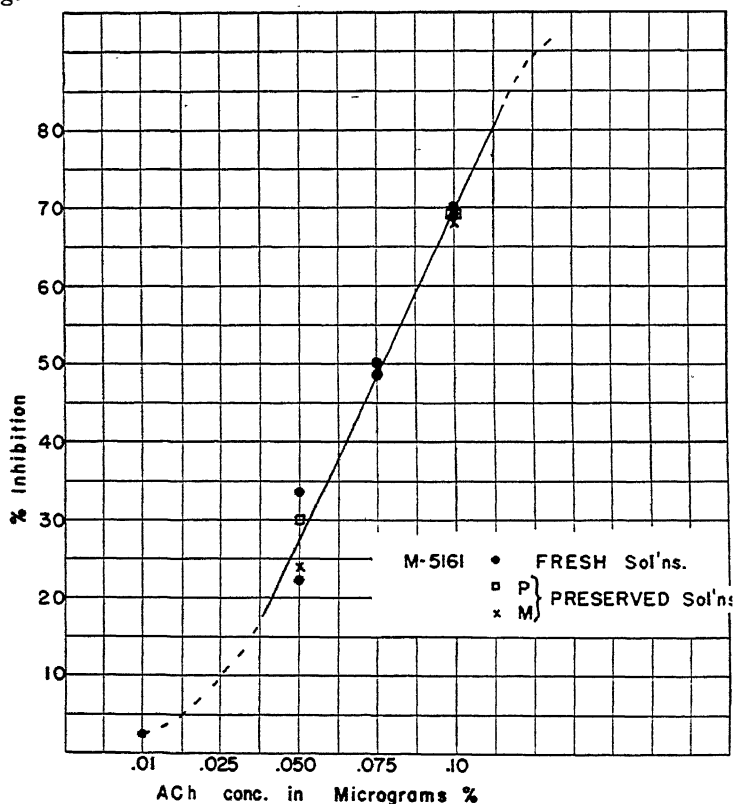
FIG. 4.   *Venus heart.   Concentration–inhibition curve.*

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THE MICRODETERMINATION OF SODIUM, POTASSIUM, CALCIUM, AND CHLORIDE IN A SINGLE PLASMA SAMPLE[1]

By John R. Polley[2]

Abstract

A procedure involving a single protein-precipitation step for the determination of sodium, potassium, calcium, and chloride in a single 0.4 ml. sample of plasma is described. The chloride and sodium methods are titrimetric, the potassium and calcium methods are colorimetric and all are accurate within ± 3%.

During the past decade, considerable interest has been focused on plasma electrolyte balances, especially in so far as these are affected by various agents such as adrenal cortical hormones (2, 3, 5). In many such studies, the levels of only one or two ions, usually sodium and potassium, are followed simultaneously. In the course of our present experiments on rats, it was considered desirable to follow the plasma levels of calcium and chloride as well as sodium and potassium for each animal. Using present analytical methods, this would require four separate plasma samples, followed by separate and different methods of protein precipitation.

The procedure presented here was developed not only to simplify the determination of these important plasma ions, but also to enable the analyses to be performed without having to sacrifice the animal to obtain sufficient plasma. By this method, which requires only 0.4 ml. of plasma, it is possible to follow at regular intervals the effect of various agents on the concentrations and ratios of these ions and to correlate these values with other findings such as blood pressure and renal clearances for each individual animal.

The analytical methods described represent adaptations of well-known procedures to a micro scale and to a common protein precipitation step. The protein-free filtrate can be stored until required for analysis. Thus, the number of animals used in an experiment need not be limited to the number that can be analyzed immediately.

All the methods involve the use of calibration curves established by analyzing known solutions of the ions by the methods described. This allows the plasma concentrations of the ions to be read directly from the curves in either milliequivalents per liter or milligram per cent,* thus eliminating the need for many calculations and for standardizing the analytical reagents. Since the reagents used are stable with ordinary precautions, except for those used in the potassium analysis, they can be made up in large quantity and the calibration curves once established are likewise constant.

* Mgm. per 100 ml.

Procedure

A blood sample of about 1 ml. is drawn from the animal by heart puncture using a 22-gauge long-bevel needle previously rinsed with an isotonic heparin solution. Since the potassium concentration within the red cells is about 20 times that in the plasma, care must be taken to prevent hemolysis, which may occur if the blood is forced through the needle or if the syringe is wet. The needle is removed and the blood is transferred to a small centrifuge tube, which is quickly stoppered, and centrifuged immediately at high speed to minimize 'chloride shift'. A 0.4 ml. sample of the plasma is added to 3.5 ml. of distilled water in a 15 ml. centrifuge tube. Then 1.0 ml. of 15% trichloroacetic acid is added, the tube is allowed to stand for 15 min. and then centrifuged. The supernatant liquid is filtered if necessary using a filter paper cut down to about 2 cm. to reduce the loss of liquid by absorption. This filtrate is stable and may be stored for future use.

CHLORIDE ANALYSIS

Reagents

1. Diphenylcarbazone indicator, 0.2%—200 mgm. of diphenylcarbazone are made up to 100 ml. with 95% alcohol. Store in the cold and prepare fresh each month.

2. Mercuric nitrate, about 0.0025 N. Dissolve 900 mgm. of reagent-grade mercuric nitrate dihydrate in 200 ml. of distilled water plus 40 ml. of $2N$ nitric acid and make up to 2 liters.

3. Sodium hydroxide, about 0.2N. Dissolve 16 gm. of reagent-grade sodium hydroxide in distilled water and dilute to 2 liters. The strength of the solution is adjusted by the addition of 5N sodium hydroxide or by dilution with distilled water so that the addition of 1 ml. just gives an end point with bromcresol green indicator when added to 1.0 ml. of protein-free filtrate plus 1 ml. of distilled water.

The chloride content is determined by a slight modification of the mercuric chloride method of Schales and Schales (6). An aliquot of the protein-free filtrate is titrated with mercuric nitrate using diphenylcarbazone as indicator. The mercuric ion reacts with the chloride ion to form mercuric chloride, which is soluble but undissociated. The indicator turns purple in the presence of an excess of free mercuric ion and gives a sharp end point.

To 1 ml. of distilled water in a 10 ml. Erlenmeyer flask add 1.0 ml. of the protein-free filtrate. Add 1 ml. of the sodium hydroxide solution. This is to neutralize the excess acidity, for although the titration can be done directly, sharper end points are obtained if the pH is about 4 to 5. Add 0.1 ml. of diphenylcarbazone indicator and titrate with mercuric nitrate to the first permanent violet color. A microburette graduated to 0.01 ml. should be used. The mercuric nitrate solution may be standardized against sodium chloride if desired but this is not necessary since the chloride content of the sample can be determined directly from a calibration curve between titration in ml. and the chloride content in milliequivalents per liter. The calibration

curve is established by analyzing a series of known sodium chloride solutions by this procedure over the range 300 to 800 mgm. %.

Analysis of known chloride solutions in triplicate by this procedure showed that the analysis is accurate within ± 2%.

SODIUM ANALYSIS

Reagents

1. Acetone wash reagent. To 2 ml. of 5% sodium chloride add 30 ml. of the uranyl zinc acetate reagent, allow to stand for 15 min. and then filter with suction. The precipitate, sodium uranyl zinc acetate, is washed with alcohol, then ether. Add this amount of the triple salt to 2 liters of acetone, shake well, allow to stand overnight, and then filter.

2. Ethyl alcohol, 95%.

3. Phenolphthalein indicator, 1%.

4. Sodium hydroxide, about $0.02N$. Dissolve 1.6 gm. of reagent-grade sodium hydroxide in distilled water and make up to 2 liters.

5. Uranyl zinc acetate reagent. Dissolve, with heating, 77 gm. of uranium acetate in about 400 ml. of distilled water containing 14 ml. of glacial acetic acid, cool and dilute to 500 ml. in a volumetric flask. In a like manner, dissolve 231 gm. of zinc acetate in distilled water containing 7 ml. of glacial acetic acid and make up to 500 ml. Mix the two solutions while hot, allow to stand for several days at about 18° C., and then filter.

The method used here is similar to that described by Weinbach (8). The sodium is determined by precipitation with uranyl zinc acetate in an alcoholic medium. The precipitate, uranyl zinc sodium acetate, is washed, dissolved in water free of carbon dioxide, and titrated with sodium hydroxide solution.

To 0.5 ml. of 95% ethyl alcohol in a 15 ml. centrifuge tube add 1.0 ml. of the protein-free filtrate. Add 5 ml. of the uranyl zinc acetate reagent and allow to stand for 30 min. Then add 0.5 ml. of 95% alcohol and stir with a small glass rod. After 30 min., add 1 ml. of 95% alcohol and again stir up the fine precipitate. After a further 60 min., centrifuge for 10 min. at high speed, decant, or preferably siphon off the supernatant, and invert the tube on a piece of filter paper to drain for about 5 min. Wash the precipitate twice by adding 5-ml. portions of the acetone wash reagent, shaking, centrifuging, and then decanting the supernatant and allowing to drain as before. The precipitate is soluble in water and is transferred quantitatively to a 50 ml. flask by washing out with 30 ml. of distilled water that has been recently boiled to free it of carbon dioxide. Add 0.1 ml. of phenolphthalein indicator and titrate with the sodium hydroxide solution to the first permanent pink color. A microburette graduated to 0.01 ml. should be used. It is not necessary to standardize the sodium hydroxide solution since it is prepared in a large quantity at the beginning of a series of experiments and the sodium content of the plasma in milliequivalents per liter is determined directly from a calibration curve between the titration of sodium hydroxide in ml. and the

corresponding sodium content in milliequivalents per liter. The calibration curve is established by analyzing known sodium chloride solutions over the range 300 to 900 mgm. % by this procedure, a 0.4 ml. sample of the standard being substituted for plasma, with the exception that the pH of the filtrate in this case should be adjusted to that of the protein-free filtrate using a pH meter and adding a few crystals of reagent-grade potassium carbonate. Plasma values read from a calibration curve established directly without this pH correction are about 5% too high. The reagents can be checked from time to time by analyzing these known solutions, which are stable, and the calibration curve corrected if necessary, although this is not likely if care is taken to keep the sodium hydroxide solution free of carbon dioxide.

Analysis of known sodium solutions in triplicate by this procedure showed that the analysis is accurate within ± 2%.

Sodium content in milliequivalents per liter

$$= \frac{\text{mgm. \% sodium chloride}}{5.85} \, .$$

Potassium Analysis

Reagents

1. Hydrochloric acid, 50%. One volume of concentrated hydrochloric acid to one volume of water.

2. *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 0.1%. Dissolve 0.1 gm. of the powder in a mixture of 90 ml. of distilled water and 10 ml. of glacial acetic acid.

3. Silver nitrate, 2%. Dissolve 2 gm. of reagent-grade silver nitrate in 98 ml. of distilled water.

4. Silver nitrate, 40%. Dissolve 40 gm. of reagent-grade silver nitrate in 60 ml. of distilled water.

5. Silver cobaltinitrite reagent. Dissolve 25 gm. of crystalline cobalt nitrate in 50 ml. of distilled water plus 12.5 ml. of glacial acetic acid. To this solution add 210 ml. of a solution of sodium nitrite prepared by dissolving 120 gm. of reagent-grade sodium nitrite in 180 ml. of distilled water. After mixing the two solutions, blow air through until all brown fumes have been driven off. This solution of sodium cobaltinitrite must be filtered each time before using. It is stable for about a month if stored in the cold. The silver cobaltinitrite is prepared fresh each day of use by adding 1 ml. of 40% silver nitrate to each 20 ml. of filtered sodium cobaltinitrite solution, shaking well, and then filtering.

6. Sodium hydroxide, 0.2N. Dissolve 16 gm. of reagent-grade sodium hydroxide in distilled water and make up to 2 liters.

7. Sulphanilamide, 0.5%. Dissolve 0.5 gm. of the pure powder in a mixture of 90 ml. of distilled water and 10 ml. of glacial acetic acid.

8. Wash reagent. Mix two parts of distilled water, two parts of 95% ethyl alcohol, and one part of ether.

The method used is a modification of that of Looney and Dyer (4). The potassium is precipitated from the protein-free and chloride-free filtrate by the addition of silver cobaltinitrite solution, which forms insoluble salts with potassium ion. The precipitate is washed free from the reagent, decomposed in hot, dilute sodium hydroxide, and the nitrite determined colorimetrically by an application of the sulphonamide method of Bratton and Marshall (1).

To 1.0 ml. of the protein-free filtrate add 0.2 ml. of 2% silver nitrate and allow to stand for 30 min. Add a small plug of glass wool to the tube and push it down to the liquid surface. Centrifuge at high speed for 10 min. Push the glass wool to the bottom of the tube with a small glass rod and then transfer 1.0 ml. of the clear filtrate to a dry centrifuge tube. The glass wool serves to push down a thin film of silver chloride that is otherwise held on the surface of the liquid by surface tension. Add 0.5 ml. of 95% alcohol and place the tube in a water bath at 10° to 15° C. for 10 min. Then add 1.5 ml. of the filtered silver cobaltinitrite reagent and leave in the water bath or in a refrigerator at not less than 5° C. overnight. A visual examination of the tubes may fail to detect much or any precipitate but after centrifuging for 15 min. at high speed, a fine yellow precipitate appears on the bottom of the tubes. The supernatant liquid is carefully removed and the precipitate washed by adding 5-ml. portions of the wash reagent, centrifuging, decanting, and allowing the tubes to drain. This procedure is repeated until the washings are clear, twice usually being sufficient. To the washed precipitate add 5 ml. of the sodium hydroxide solution, break up the precipitate with a small glass rod, and place the tubes in a boiling water bath for five minutes. After cooling the tubes, transfer the contents quantitatively with washings with 50 ml. of water to a 250 ml. volumetric flask. Add 1 ml. of 50% hydrochloric acid and 2 ml. of 0.5% sulphanilamide solution. Mix thoroughly and after three minutes, add 1.5 ml. of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride solution. After five minutes dilute with water to the 250 ml. mark, transfer a sample to a colorimeter tube, and read in a photoelectric colorimeter using a 525 mμ filter. For a blank use a sample from a 250 ml. flask treated by the same procedure beginning with the addition of 5 ml. of alkali to a dry centrifuge tube. The potassium content is read from a calibration curve between colorimeter readings and potassium content established by treating known solutions of potassium chloride over the range 5 to 30 mgm. % of potassium (9.55 to 57.31 mgm. % potassium chloride) by this procedure. It has been found advisable to run a set of standards with each analysis since the calibration curve is not constant from day to day, apparently varying slightly with the freshness of the precipitating reagent and the time and temperature of standing.

Analysis of known potassium solutions in triplicate by this procedure showed that the analysis is accurate within ± 3%.

CALCIUM ANALYSIS

Reagents

1. Ammonium hydroxide, about $1N$. Dilute 7 ml. of reagent-grade concentrated ammonium hydroxide to 100 ml. The strength of this solution is adjusted so that the addition of 0.2 ml. of it to 1.0 ml. of protein-free filtrate just gives an end point with bromcresol green indicator.

2. Ammonium hydroxide, 2%. Dilute 2 ml. of concentrated ammonium hydroxide to 100 ml.

3. Ammonium oxalate, saturated. Reagent-grade ammonium oxalate is dissolved to saturation (about 4%) in distilled water at room temperature.

4. Ceric sulphate, $0.1N$. Dissolve 4 gm. of ceric sulphate in $1N$ sulphuric acid to make 100 ml. of solution. Store in a dark bottle.

5. Ceric sulphate, $0.001N$. Dilute 1 ml. of the $0.1N$ ceric sulphate to 100 ml. with $0.1N$ sulphuric acid. This solution is prepared fresh for use.

6. Potassium iodide, 1%. Dissolve 1 gm. of reagent-grade potassium iodide in 99 ml. of distilled water. This solution is prepared fresh for use.

7. Sulphuric acid, $1N$. Add 27 ml. of reagent-grade concentrated sulphuric acid to about 500 ml. of distilled water and then dilute to 1 liter.

8. Sulphuric acid, $0.2N$. Dilute 200 ml. of the $1N$ sulphuric acid to 1 liter with distilled water.

9. Sulphuric acid, $0.1N$. Dilute 100 ml. of the $1N$ sulphuric acid to 1 liter with distilled water.

10. Wash reagent. Mix two parts of distilled water, two parts of 95% ethyl alcohol, and one part of ether.

This procedure is a modification of that described by Sendroy (7). The calcium is precipitated from the protein-free filtrate as calcium oxalate, which is washed, dried, and then dissolved in acid. A constant amount of ceric sulphate solution in slight excess is added and then the unreduced ceric sulphate determined by iodometry, the yellow color being read in a photo-electric colorimeter.

In this analysis, the plasma content of the sample is read from a calibration curve established by analyzing solutions of known calcium content over the range 6 to 15 mgm. % of calcium (15.0 to 37.5 mgm. % calcium carbonate) by this procedure and the set of standards is run at the same time as the experimental samples. The standard calcium solutions are treated in exactly the same manner as a 0.4 ml. sample of plasma.

To a calibrated 15 ml. centrifuge tube is added 1.0 ml. of the protein-free filtrate and the tube is placed in a water bath at 70° C. for five minutes. Then 1 ml. of saturated ammonium oxalate solution is added and the tube left in the water bath for 20 min. The tube is removed from the bath and 0.2 ml. of the ammonium hydroxide reagent is added. After being allowed to stand overnight at room temperature, it is centrifuged at high speed for 10 min. All but about 0.2 ml. of the supernatant is siphoned off using a capillary tube

with an upturned tip to avoid disturbing the precipitate, which is then washed by adding 2 ml. of 2% ammonium hydroxide, centrifuging, and siphoning off the supernatant as before. The precipitate is washed twice more with 2-ml. portions of the wash reagent. The tube is then dried in an oven at 100° C. for 30 min., then 2.0 ml. of 0.2N sulphuric acid is added, and the tube placed in a water bath at 70° C. for five minutes. To prepare a reagent blank, 2.0 ml. of 0.2N sulphuric acid is added to a clean dry tube, which is also placed in the water bath. At the end of five minutes, the tubes are removed and allowed to cool. Then 3.0 ml. of 0.001N ceric sulphate solution are added to all the tubes except the reagent blank tube. To the tube of reagent blank are added 3.0 ml. of distilled water instead of the ceric sulphate solution. The tubes are placed in the water bath at 70° C. for 10 min. after which they are removed, allowed to cool, and then 1.0 ml. of 1% potassium iodide is added to each. After one minute the volume in the tubes is made up to 6 ml. with distilled water. The contents of the tubes are transferred to micro-colorimeter tubes and read in a photoelectric colorimeter using a 400 mμ filter. The colorimeter tube containing the reagent blank is placed in the instrument and the needle adjusted to zero. The remaining tubes are then read in the ordinary way. The calcium content of the plasma sample is read from a calibration curve between colorimeter readings and known calcium content of the standards run at the same time as the experimental samples.

Analysis of known calcium solutions in triplicate by this procedure showed that the analysis is accurate within ± 3%.

To demonstrate the precision of the four analyses described above, the same blood sample was analyzed five times by these methods and the results are shown in Table I.

TABLE I

Analysis No.	Sodium, m. e./liter	Chloride, m. e./liter	Potassium, m. e./liter	Calcium, m. e./liter
1	145.2	103.8	5.5	6.2
2	144.2	103.1	5.2	6.5
3	143.8	102.8	5.0	6.5
4	146.4	104.4	5.8	6.7
5	147.0	104.6	5.7	6.9
Average	145.3	103.7	5.4	6.6
Standard deviation	±1.4	±0.8	±0.3	±0.3

The plasma obtained from a series of normal male white rats maintained on Purina Fox Chow and tap water ad libitum was analyzed by this procedure. Eight typical results selected at random from this series are presented in Table II.

TABLE II

PLASMA CONCENTRATIONS OF SODIUM, POTASSIUM, CHLORIDE, AND CALCIUM

No.	Wt.	Chloride, m. e./liter	Sodium, m. e./liter	Potassium, m. e./liter	Calcium, m. e./liter
1	189	106.8	142.5	7.0	6.2
2	179	104.5	143.6	4.9	6.2
3	190	102.5	144.2	5.8	6.5
4	182	104.5	142.5	4.9	6.5
5	182	104.5	144.2	5.5	6.2
6	175	103.6	150.4 ●	4.2	6.5
7	142	103.8	145.3	6.0	6.7
8	176	104.1	140.5	6.2	7.0
Average		104.3	144.1	5.5	6.5
Standard deviation		±1.2	±2.9	±0.9	±0.3

Acknowledgment

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A METHOD OF TYPING *HAEMOPHILUS INFLUENZAE* BY THE PRECIPITIN REACTION[1]

By CATHERINE F. C. MACPHERSON[2]

Abstract

A method of typing *H. influenzae* by the precipitin reaction is described. The procedure consists of dissolving the bacterial culture in 90% phenol to destroy the specificity of the somatic proteins followed by alcoholic precipitation of the denatured proteins and type specific polysaccharide. The carbohydrate is extracted from the precipitate with saline and portions of the saline extract added to samples of the six type specific antisera. A positive test is indicated only by a marked turbidity, which develops within five minutes after mixing and denotes the extraction of at least 0.01 mgm. of polysaccharide. A strong positive test is, of course, regularly obtained in the case of cultures shown to be encapsulated by the usual capsular swelling technique. However, the method was devised as an attempt to ascertain whether non-typable (by the capsular swelling technique) derivatives of strains of known type or non-typable, but suspected respiratory pathogens, isolated from clinical material, contained detectable quantities of any of the known type specific polysaccharides.

Introduction

There are now six recognized types of pathogenic *Haemophilus influenzae* (5). These pathogenic strains, in contrast to the nonpathogenic forms, produce an iridescent growth on Levinthal or Fildes agar and have a well defined capsule. The specific capsular substances of Types *a*, *b*, *c*, *d*, and *f* have been shown to be polysaccharides. Some of the chemical, physical, and immunochemical properties of these carbohydrates have been recorded (4).

In the course of immunochemical studies on the somatic proteins of *H. influenzae*, it was noted that extracts of non-encapsulated derivatives of Type *b* meningeal strains contained appreciable quantities of Type *b* polysaccharide. One derivative strain that was not iridescent and not encapsulated has been subcultured repeatedly on Fildes agar over a period of three years and continued regularly to produce the capsular substance.

The frequency with which one encounters colonies of non-encapsulated organisms in cultures of pathogenic *H. influenzae* under laboratory conditions subsequent to isolation from clinical material is well known. Thus it was considered that a method by which minute amounts of capsular substance could be isolated and identified by the precipitin reaction would be useful as

an adjunct to the capsular swelling technique in identifying a poorly encapsulated strain as well as in ascertaining the origin of non-encapsulated derivative strains in which the transition from S $\longrightarrow$ R was not complete.

Method

The method finally devised is as follows. To the yield of heavy, confluent 18 hr. growth from one Fildes or Levinthal agar plate suspended in 1.5 ml. of 1 M sodium acetate are added five volumes of 95% ethyl alcohol. After centrifugation, the supernatant is discarded, the precipitate (measuring about 0.15 ml. in volume) smoothed out and next dissolved by the addition of 1.0 ml. of 90% phenol for 15 min. Phenol denatures the bacterial proteins and renders them insoluble in saline but does not injure the specific polysaccharides. Then 0.1 ml. of 20% sodium acetate and 5 ml. of chilled ethyl alcohol are added with thorough mixing and the tubes left at 0° C. for at least one hour. The polysaccharides are less soluble in alcohol at 0° C. than at room temperature. Thus for good recovery of polysaccharide it is advisable to maintain all alcoholic mixtures at this lower temperature. The bulky precipitate of denatured protein and polysaccharide is centrifuged down, washed twice with 2 ml. portions of chilled alcohol to remove all but traces of phenol, and finally extracted with 1.0 ml. of saline. To perform the test, 0.1 ml. of clear extract is added to 0.1 ml. portions of each of the six type specific antisera in 50 × 6 mm. tubes and the tubes are read within five minutes after mixing. The antisera should contain at least 0.3 mgm. of precipitin nitrogen per ml. (1, 2, 3, 4). Only immediate marked turbidity is considered as a positive test and this depends upon the extraction of about 0.01 mgm. of polysaccharide.

After 15 to 30 min. a slight cloudiness develops in those tubes that contain anti *c, d, e,* or *f* antisera. This precipitation is due to a reaction between a species specific protein, which escapes denaturation by phenol and the species specific antibody that comprises a relatively large proportion of the total antibody in Types *c, d, e,* and *f* antisera.

Results

At least one non-typable variant of each of the six kinds of encapsulated influenza strains has been examined by this method. The Type *b* variants regularly gave a positive test for type specific carbohydrate whereas Type *a, c, d, e,* and *f* variants usually yielded negative or weakly positive results. Studies are in progress on additional derivatives of encapsulated strains.

The knowledge that some non-encapsulated strains that were variants of known encapsulated strains contained detectable amounts of type specific polysaccharide suggested that the method described above might be used to throw some light on the question as to whether or not the large numbers of non-encapsulated, non-iridescent strains of *H. influenzae* that are often isolated from clinical material are derived from encapsulated strains.

To date the method has been applied to a group of 20 such strains. Ten of these were isolated from normal throats and 10 from cases of respiratory infections. In all, the results were negative.

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THE INHIBITION OF HYALURONIDASE BY SODIUM SALICYLATE AND ITS POSSIBLE METABOLITES[1]

By Julius Lowenthal[2] and Arthur Gagnon[3]

Abstract

It has been shown that salicylic acid and gentisic acid, a substance formed in the organism from salicylic acid, have no influence on the activity of hyaluronidase *in vitro*. However carboxy-*p*-benzoquinone, the quinone corresponding to gentisic acid, was found to inhibit the enzyme *in vitro*.

Salicylic acid and its derivatives have been used for more than 50 years in the treatment of rheumatic fever. Despite the fact that during this period salicylates have been one of the most frequently investigated drugs, the mechanism underlying their therapeutic effects has remained obscure. This state of affairs has been due, to a large extent, to our ignorance of the etiology of rheumatic fever, and this naturally has had a bearing on all these investigations.

Recently a number of facts have become known that seem to give some indications as to the nature of rheumatic fever, as well as the mode of action of salicylates in the treatment of this disease.

It has been known for some time that extracts from such sources as invasive bacteria (like hemolytic streptococci Group A (5, 16)), testes (12), poisonous insects, and snake venoms (7) increase the permeability of the skin. This can be demonstrated by injecting these extracts (spreading factors) together with suitable indicators (India ink) intradermally in experimental animals and comparing the area of diffusion with that of the simultaneously injected indicator minus the spreading factor. Working along chemical lines, a number of investigators have been able to isolate from these spreading factors an enzyme, hyaluronidase, which acts specifically on a mucopolysaccharide, hyaluronic acid (20, 3). This polysaccharide is found in the skin (18), umbilical cord, synovial fluid (19), and other sources, and more generally seems to form an essential constituent of the connective tissue. When the action of the enzyme on its substrate is followed *in vitro*, there is first a depolymerization, as can be seen by a decrease in the viscosity and by the loss of the protein precipitating power of hyaluronic acid. This is followed later by complete hydrolysis, as evidenced by increased reducing power. It is precisely in those regions rich in hyaluronic acid that the swelling and lesions, so characteristic of rheumatic fever, are known to occur. Furthermore, it has been

observed for some time that rheumatic fever is frequently preceded by streptococcal infections of the throat, from which organism hyaluronidase has been extracted.

Since it is generally held to-day that no causative bacterial agent has ever been isolated from the affected area in rheumatic fever, a possible causal relationship between hyaluronidase and rheumatic fever may tentatively be inferred.

A recent experiment by Guerra (9) has been the starting point for the present work, and seems to be of great significance when viewed in relation to the above-mentioned facts. When hyaluronidase together with India ink was injected into the skin of albino rabbits or human subjects after the intravenous injection of sodium salicylate, there was an inhibition of the spreading. It would appear that this action is specific for salicylates, for when sulphadiazine was tested, no such inhibition was observed. On the basis of Guerra's work, we concluded that if this decrease in diffusion was due to an inhibition of the enzyme hyaluronidase by the salicylate or a product formed from it in the organism, it would perhaps be possible to reproduce this inhibition *in vitro* and in this way obtain more definite information on the mode of action of salicylates in rheumatic fever.

Experimental

Preparation of Test Substances

Extraction of Hyaluronidase from Bovine Testes

The method followed for the extraction of hyaluronidase was essentially that of Hahn (11). The precipitate obtained at 70% ammonium sulphate saturation was suspended in a small amount of water and dialyzed against running water until salt free; after reducing the volume by distillation under reduced pressure at a maximum temperature of 30°, the solution was evaporated to dryness while freezing.

Hyaluronic acid was obtained through the courtesy of Dr. E. Schwenk, Schering Corporation.

The viscosimetric method of following the enzymatic depolymerization of hyaluronic acid by the decrease of the viscosity was used. The experimental arrangement is similar to that of Haas (10) with slight changes in the concentration of enzyme and polysaccharide and in buffer solutions. It is shown in Table I.

Method of calculating the specific viscosity and inhibition

t = time after the addition of hyaluronidase

t_1 = flow time of solution containing buffer enzyme and polysaccharide

t_2 = flow time of solution containing solvents

t_3 = reaction time = $t + 0.5\ t_1$

η = specific viscosity = $\dfrac{t_1}{t_2} - 1$

R_0 = time to reach half viscosity without inhibitor

R = time to reach half viscosity with inhibitor

I = % of inhibition = $\left(1 - \dfrac{R_0}{R}\right)$

TABLE I

	Expt. I	Expt. II	Expt. III	Expt. IV	Expt. V	Expt. VI
0.9% NaCl, ml.	1.0	1.0	1.0	1.0	1.0	1.0
Hyaluronidase, mgm. (dissolved in 1 ml. 0.9% NaCl)	—	—	.125	.125	.125	.125
0.5M phosphate buffer, pH 7, ml.	1.0	1.0	1.0	—	—	—
1 ml. carboxy-p-benzoquinone (dissolved in 0.5M phosphate buffer, pH 7), concentration, moles/liter	—	—	—	1×10^{-3}	2.5×10^{-3}	5×10^{-3}

Incubated separately for 10 min. at 26° C.

	Expt. I	Expt. II	Expt. III	Expt. IV	Expt. V	Expt. VI
0.02 M acetate buffer, pH 4.7, ml.	2.0	—	—	—	—	—
Polysaccharide (dissolved in 0.02M acetate buffer, pH 4.7) concentration, 2 mgm./ml. : ml.	—	2.0	2.0	2.0	2.0	2.0
Flow time, sec.	54.3	127.7				
% Inhibition = $100\left(1 - \dfrac{R_1}{R}\right)$				40.2	60.4	68.2

When sodium salicylate was added to the system in concentration of $10^{-2}M$ and in even higher concentrations, no inhibition was observed. Since this research was started, a number of publications have come to our attention where the same problem was investigated. Pike (22) and Meyer (17) have stated that they failed to observe any inhibition *in vitro* of hyaluronidase by sodium salicylate, while Dorfmann, Reimers, and Ott (6) and Calesnick and Beutner (2) have reported that they were able to produce inhibition by sodium salicylate. Since salicylic acid in higher concentrations is known to be a flocculating and precipitating agent of proteins, it may be that the inactivation observed might have been due to this effect, rather than to a specific inhibition (13).

We concluded, after the negative result with salicylate, that the effect observed by Guerra might have been due to a compound formed from salicylic acid in the organism. One of the outstanding facts in the salicylate therapy of rheumatic fever are the large doses used. Concentrations of 35 to 50 mgm. per ml. of blood has been advocated (4). Studies on the fate

of the compound in the organism show that about 80% is excreted in forms containing intact salicyl groups, while 4 to 8% is converted to gentisic (2,5-dihydroxybenzoic acid) and related compounds (14). The latter seems to be the only new compound formed, since the formation of conjugated product may be considered a general mode of detoxification.
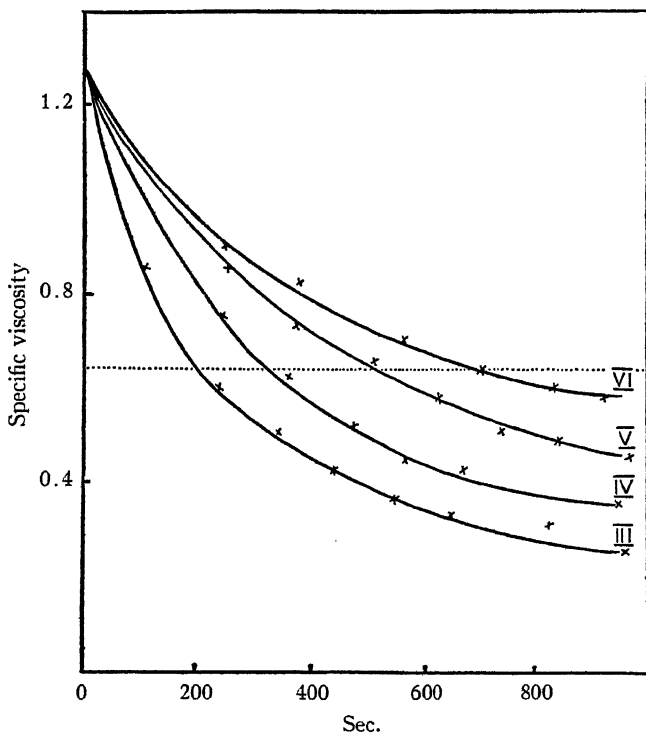
FIG. 1.

We therefore tested the activity of gentisic acid in the same way as that of its precursor, salicylic acid, and this compound was also found to be inactive. Since gentisic acid is a p-diphenol, it may be susceptible to further oxidation, the next product of oxidation being the corresponding quinone. In a model experiment with p-benzoquinone, a significant inhibition was obtained.

A survey of the literature (21, 23, 2) indicates that several attempts to prepare the quinone corresponding to gentisic acid were unsuccessful. We first tried to obtain this compound by the enzymatic oxidation of gentisic acid. Incubation with peroxidase and polyphenol oxidase showed that gentisic acid was acted upon by these enzymes, but no active substance could be extracted from these mixtures. We finally succeeded in finding a method for the chemical oxidation of gentisic acid to its quinone and this product proved effective as an inhibiting agent, at a concentration of $10^{-3}M$ and higher.

Syntheses

Gentisic acid was prepared after the method of Mauthner (15). It was purified by precipitation from ether with petrol ether (fraction 65° to 95° C.). It gave a fusion point of 204° C. and with ferric chloride a characteristic corn-blue coloration.

Carboxy-*p*-benzoquinone. A method described by Thiele (24) for the preparation of similar unstable quinones by dry oxidation with nitric oxides was used in the preparation of this compound, after attempts to prepare it by other methods failed. Finely powdered gentisic acid is placed on a watch glass in a desiccator over fuming nitric acid. Partial evacuation aids in the evolution of nitric oxides. When the reaction is finished the crystals are placed over soda lime in a vacuum to take off any nitric oxides. The crystalline mass was dissolved in ether, and after filtration and evaporation of the ether, a reddish brown granular mass was obtained. Attempts to find more suitable solvents for obtaining the quinone in a crystalline form were unsuccessful. The compound decomposed without a sharp melting point, and with 2,4-dinitrophenylhydrazine, it yielded a compound that melted at 182° C. It is probable that the quinone was not pure, but still contained unreacted gentisic acid.

General Observations

In discussing the possible significance of the inhibition of hyaluronidase *in vitro* by carboxy-*p*-benzoquinone, it must be borne in mind that the spreading reaction itself is a complex phenomenon (8), depending on many factors, such as age, sex hormones, etc. When the enzymatic activity is followed *in vitro*, conditions are so different that it is hardly warranted to make any definite statement as to the possibility of corresponding mechanism *in vivo*, and carboxy-*p*-benzoquinone has not yet been shown to be formed in the organism after the administration of salicylic acid, although its formation may seem possible.

Moreover, the experimental arrangement in the viscosimetric measurement is such that it is very difficult to draw any definite conclusions as to whether the observed effect is due to a specific inhibition, or to an unspecific denaturation of the enzyme. However, the low concentration of the quinone makes this point unlikely.

We therefore think that it would need further experimental evidence, to correlate our findings with the actual mode of action of salicylates in rheumatic fever. Investigations planned to this end are in progress in our laboratory.

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GLYCOSURIA IN PHLORIZINIZED RATS DEPLETED OF PYRIDOXINE[1]

By Edouard Pagé[2] and R. Gingras[3]

Abstract

Glucose excretion and dextrose : nitrogen ratios were measured following phlorizination in young fasting rats depleted of pyridoxine and in their pair-fed controls. The same determinations were also made in older and non-fasting animals following a period of 11 to 16 weeks on a sugar-free, pyridoxine-free ration. Pair-fed rats were again used as controls. No significant differences were found between groups. It is concluded that under the experimental conditions described, pyridoxine insufficiency does not exert a specific effect on gluconeogenesis.

Introduction

The members of the vitamin B₆ group have been shown by Gale and Epps (3) and by Gunsalus and Bellamy (4) to be converted into the coenzyme of amino acid decarboxylases. The importance of these vitamins in transamination reactions has been demonstrated further by Schlenk and Snell (12) and by Lichstein *et al.* (8). In 1941, McHenry and Gavin (9) had already suggested that pyridoxine was essential for the metabolism of protein. Cerecedo and Foy (2) then found that pyridoxine depletion in rats could be accelerated by maintenance of the animals on a high protein diet. This close relationship between pyridoxine and amino acid metabolism suggests that gluconeogenesis might be affected by a lack of this vitamin. D/N ratios were therefore determined in phlorizinized rats that had previously been depleted of pyridoxine. Bartlett and Gaebler (1) have recently reported that the low fasting D/N ratio of a depleted dog could be raised by the administration of pyridoxine. In another instance, desoxypyridoxine seemed to contribute to a low D/N ratio in a dog receiving a sugar-free ration.

Experimental

In a first experiment, young albino rats averaging 53 gm. body weight were put on the following ration: sucrose, 72; "Vitamin Test" casein (Smaco), 18; corn oil (Mazola), 4; Salt Mixture, 4; and Cellu flour, 2 parts. One hundred grams of ration contained: thiamine hydrochloride, 0.4; riboflavin, 0.4; pyridoxine hydrochloride, 0.0 or 0.5; calcium pantothenate, 3.0; nicotinic acid, 3.0; inositol, 10; 2-methyl-1,4-naphthoquinone, 0.1; and choline chloride, 150 μgm. The rats also received two drops weekly of a fish liver oil concentrate enriched with 0.5 μgm. of α-tocopherol per drop. The salt mixture

consisted of U.S.P. Salt Mixture XII, No. 2, to which had been added the following: potassium iodide, 0.7; copper sulphate pentahydrate, 0.3; manganese sulphate tetrahydrate, 5.0; and zinc carbonate, 0.2 gm. per kilogram of salt mixture.

Litter mates were paired according to weight and one rat of each pair received the full ration while the other received the pyridoxine-free diet. The food was weighed each day and the consumption of the control animal was limited to that of his mate on the depletion ration. After six weeks, measurements were begun and the last pairs of rats to be tested had been on the above rations for nine weeks. The rats were given subcutaneously 50 μgm. of phlorizin dissolved in 0.5 cc. of sesame oil on the first and second mornings of a two day fast. The urine was collected under light mineral oil in the presence of a preservative and determinations were made on each of the two 24 hr. samples. Glucose was determined by the Shaffer–Hartmann method (6) and nitrogen by micro-Kjeldahl digestion, distillation of the ammonia into boric acid, and titration. Ketone bodies were measured by the Van Slyke gravimetric method (6).

The rats were greatly emaciated at the time measurements were begun and several did not survive the two day fast. Results are given in Table I for the 13 pairs of rats where both members survived. While the data for the other pairs were incomplete, they were similar to those given here.

TABLE I

GLYCOSURIA AND KETONURIA IN PYRIDOXINE DEPLETED RATS AND THEIR PAIR-FED CONTROLS
(13 pairs)

	Depleted	Controls
Average length of depletion period (days)	52	
Average final weight at the time of phlorizination (gm.)	109 $\pm$ 6*	118 $\pm$ 7
Glucose excretion, first 24 hr. (μgm. per 100 gm.)	557 $\pm$ 56	400 $\pm$ 94
Glucose excretion, second 24 hr. (μgm. per 100 gm.)	284 $\pm$ 34	276 $\pm$ 52
D/N ratios, second 24 hr.	2.82 $\pm$ 0.14	2.67 $\pm$ 0.17
Ketone bodies, second 24 hr. (μgm. per 100 gm.)	116 $\pm$ 24	71 $\pm$ 13

* *Standard deviation of the mean.*

In a second experiment, 20 male rats averaging 190 gm. body weight were used. These rats had previously been on a purified diet containing 30% casein. They were in good health and had made average weekly gains of 30 gm. in the preceding five weeks (since weaning). They were put on a sugar-free ration of the following composition: "Vitamin Test" casein (Smaco), 72; mineral mixture, 4; soybean oil, 20; Cellu flour, 4 parts. One hundred grams of ration contained: thiamine hydrochloride, 0.4; riboflavin, 0.4;

pyridoxine, 1.0; calcium pantothenate, 3.0; nicotinic acid, 3.0; inositol, 10; 2-methyl-1,4-naphthoquinone, 0.1; and choline chloride, 150.0 μgm. The mineral mixture was the same as that used previously and a fish liver oil concentrate fortified with α-tocopherol was also given.

Following three weeks on this regimen, the rats were divided into eight pairs for paired feeding purposes, one rat remaining on the basal ration and the other receiving that ration less its pyridoxine. The remaining four rats were fed *ad libitum*.

After periods of time varying between 77 and 113 days, the D/N ratios were measured on the non-fasting animals. They received each morning a subcutaneous injection of 30 μgm. of phlorizin in sesame oil. Immediately thereafter 24 hr. collections of urine were started. The control rats were injected one day after the depleted ones so that the glucose and nitrogen excretions should correspond to similar food intakes for each rat of a pair. Before any depleted rat was phlorizinized, it was ascertained that a urine sample gave a positive test for xanthurenic acid as evidenced by the appearance of a dark green color when ferric chloride was added to the neutralized urine (7).

Fig. 1 shows the changes in body weight of the three groups from the time the animals were paired until measurements were begun. During the three weeks preceding pairing, the animals had first lost a little weight and then regained it. It is thus seen that the rats fed *ad libitum* remained at an almost stationary weight for some seven weeks before resuming growth.
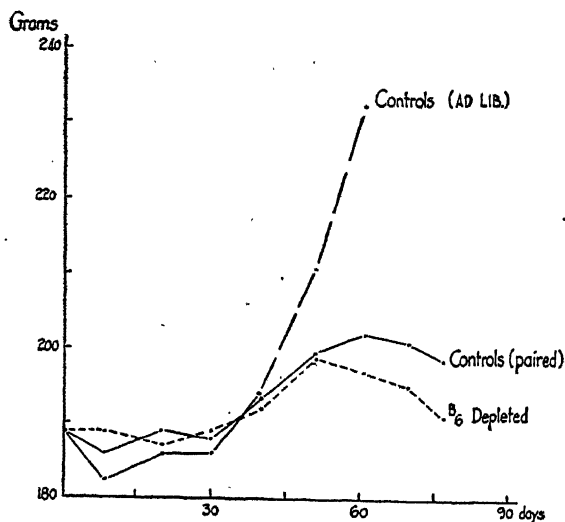
FIG. 1

Table II shows the results of the glucose and nitrogen determinations as well as other pertinent data.

TABLE II

GLYCOSURIA IN PYRIDOXINE DEPLETED RATS AND THEIR PAIR-FED CONTROLS

Pair No.	Depletion period, days	Depleted or controls	Final body weight	Changes in body weight, gm.		D/N ratios		Glucose excretion, μgm.	
						1st day	2nd day	1st day	2nd day
1	77	D	202	−30*	−36**	2.67	1.88	1600	1294
		C	237	1	−5	2.41	2.62	1780	2212
2	77	D	196	0	−30	2.97	3.28	2122	1188
		C	192	−4	−24	2.91	3.08	2020	940
3	84	D	180	−3	−9	2.85	3.20	1546	980
		C	204	21	−7	3.15	3.41	1652	1050
4	84	D	183	12	−1	3.32	3.48	2150	1032
		C	180	10	−3	2.92	3.49	1733	558
5	91	D	195	8	−6	3.34	3.45	1498	918
		C	213	23	0	3.63	3.30	997	1102
6	91	D	142	2	2	2.97	2.15	1652	858
		C	122	−22	−22	2.04	2.36	983	1139
7	113	D	220	14	−22	2.96	2.45	2432	1968
		C	237	33	−4	2.81	2.79	1946	1901
8	113	D	182	−15	−30	3.31	2.70	1865	864
		C	199	7	−29	3.05	2.30	2010	1021
Averages		D	188	−2	−17	3.05	2.82	1858	1138
		C	198	8	12	2.87	2.92	1640	1240

* From initial body weight.
** From highest weight reached.

Discussion

It has been shown by other workers (2, 10) that rats can survive for weeks and even months on a pyridoxine-free ration and it is difficult to estimate the degree of depletion in this vitamin over relatively short periods of time.   One may also question which degree of vitamin insufficiency is best suited for the study of metabolic disturbances resulting directly therefrom.   It is clear that should the condition proceed until the onset of severe clinical symptoms, all metabolic functions may be so impaired directly or indirectly as to confuse the issue.   In the present experiments, measurements were made at a time when the rats were considered to be moderately deficient in pyridoxine. Paired feeding was deemed essential to distinguish the specific effects of a lack of pyridoxine from indirect ones resulting from general undernutrition or an unsuspected lack of some other factor.

In the first experiment, where young rats were used, signs such as rustiness of the fur and acrodynia were observed in addition to the reduced growth rate. The fact that several rats did not survive a two day fast in a diabetic condition would indicate that the degree of inanition was as advanced as the

subsequent experimental procedure would allow.   When older rats were used, it is inferred that they had a large reserve of pyridoxine at the beginning of the depletion period since they had been previously on a high protein, high pyridoxine diet, two conditions favoring a stocking of the vitamin in the tissues (13, 14).   It may be seen from Fig. 1 that the lack of pyridoxine did not make itself felt on the body weights for about six weeks, the rats fed *ad libitum* behaving similarly until then.   By the time measurements were made, however, all the depleted rats had reached a plateau or were losing weight. , Their appearance was much worse than would be deduced from the weight changes and it is likely that they grew in length while losing their fat reserves.   They were greatly emaciated and had an unthrifty-looking fur.

The differences in body weight between the depleted rats and their pair-fed controls are not statistically significant.

We are at a loss to explain the sudden resumption of growth of the rats fed *ad libitum* or the similar tendency evidenced by the others at the same time and on a separately prepared ration (Fig. 1).

Widely fluctuating values were found in the first experiment with no significant differences between rats of the same pair except in respect of ketonuria, which was somewhat higher in the depleted animals.   The high excretion of glucose by the depleted rats on the first day of fast (Table I) may be due to the fact that these rats ate at leisure during the day and night preceding phlorizination while their mates, which usually ate ravenously upon being fed their quota, had probably been fasting since their last feeding 24 hr. before. It is thought that some of the low D/N ratios may have been due to inanition. Values above 3.0 have been found by us for thriving rats during fast.

In the second experiment, we likewise find no difference in the D/N ratios between depleted and control animals.   Several values are rather low, the two lowest recorded being given by the two rats who had lost the most weight since the beginning.   One was a depleted rat (Pair 1) and the other was a control rat (Pair 6).   Phlorizination for a longer period of time might have revealed some differences between groups.   On the other hand, it is interesting to note that although these rats had not eaten any sugar for 11 to 16 weeks, the depleted ones excreted at least as much glucose as their controls on the first day following phlorizination.   These initial values may be more indicative of the physiological state of these animals than those that would result from prolonged experimental diabetes when metabolism is of necessity more perturbed.

On the basis of the above findings, it is concluded that pyridoxine does not exert a specific effect on gluconeogenesis.   Such a conclusion, however, is difficult to reconcile with the statement that this vitamin is directly concerned in fat synthesis from proteins (9) since it is generally believed that such a synthesis is effected by the glucose (or pyruvic acid) pathway (11, p. 446; 5). One would expect any interference with this process resulting from pyridoxine insufficiency to manifest itself before the amino acids have reached the glucose stage.

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THE DIET AND HORMONALLY INDUCED NEPHROSCLEROSIS[1]

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Abstract

Kidney lesions resembling those of chronic nephritis and nephrosclerosis developed in rats treated with L.A.P. (lyophilized anterior pituitary) and fed 'Purina', but did not develop in similar rats similarly treated and fed Purina mixed with cornstarch in a 1 : 1 ratio. When synthetic diets, differing only in their relative carbohydrate and protein content, were fed, only those L.A.P.-treated rats that consumed a regimen containing 30% casein and 54% cornstarch developed nephrosclerosis. No such lesions were seen in treated rats kept on a 15% casein and 69% cornstarch diet. This difference in response is due to the protein and not to the cornstarch content of these diets; the entire amount of cornstarch was substituted by wheat starch or 15% of it was replaced by an equicaloric amount of fat, without influencing the development of kidney lesions.

Various protein preparations differed in their ability to cause kidney damage. Casein, egg albumen, and wheat gluten were more damaging than lactalbumin, gelatin, or zein.

The severity of nephrosclerosis caused by L.A.P. roughly paralleled the kidney hypertrophy, adrenal enlargement, and increased appetite for water, but not necessarily the food intake or the growth rate. The appearance of these renal lesions was not associated with any gross change in urine pH, but was preceded by a marked albuminuria. By the fifth day, large amounts of albumin were present in the urine of the majority of treated rats consuming the 30% casein diet. Albumin rarely appeared in the urine of treated rats consuming the 15% casein diet.

These findings were discussed in relation to the literature concerning the dietary production of chronic nephritis. The procedure described in this paper greatly accelerates the development of kidney lesions, apparently identical with those shown previously to ensue on the feeding of high protein diets.

Introduction

It has been amply demonstrated that the chronic treatment of rats with desoxycorticosterone acetate (D.C.A.), or crude cattle anterior-pituitary preparations, is followed by the development of kidney lesions similar to those seen in malignant hypertension (45-48). These lesions are characterized by thickening and necrosis of arteriolar walls, enlargement and hyalinization of glomeruli and the formation of hyaline casts with resultant marked tubular dilatation. D.C.A. produces discrete lesions in six to eight weeks in normal rats, but partial nephrectomy and a high salt intake greatly aggravate and accelerate the response (48, 49). Similarly, a high salt intake and partial nephrectomy sensitize the rat to the nephrosclerotic activity of anterior-pituitary preparations (16). Other dietary factors are also of importance (5)

since rats consuming 'Purina Fox Chow'* and treated with crude anterior-pituitary, develop severe kidney lesions, while similar rats similarly treated but fed 'Pablum'**, remain completely free.   In the present communication a report is given of a series of experiments designed to determine to what dietary factor or factors this marked difference in response could be attributed.

Experimental

The reported composition of 'Purina' differs greatly from that of Pablum both in the types and in the proportions of food materials used in its preparation.   For example, there is a marked difference in the relative amounts of carbohydrate and protein present.   Purina contains approximately 50% carbohydrate and 26% protein, while Pablum contains about 70 and 15%, respectively.   By adding cornstarch to Purina it is possible to prepare a mixture of approximately the same carbohydrate–protein ratio as Pablum. The first experiment was performed to determine whether such a change in the Purina diet would influence the nephrosclerotic activity of lyophilized beef anterior-pituitary (L.A.P.)***.

The Nephrosclerotic Activity of L.A.P. in Rats Consuming a Modified Purina Diet

Procedure: Four groups of 10 male piebald rats, weighing 40 to 60 gm. (average 51 gm.), were castrated, partially nephrectomized, and given 1% sodium chloride to drink.   One group was fed ground Purina, a second, 50% Purina, and 50% cornstarch.   The two remaining groups were fed these diets and were injected subcutaneously twice daily with 10 mgm. of L.A.P.   The diets were prepared daily by mixing with enough water to form firm cakes. After 26 days of treatment, all surviving rats were sacrificed.

At autopsy, the kidneys and adrenals were fixed in 'Suza' for 24 hr., then transferred to 4% formalin.   They were then dissected and weighed on an analytical balance;   kidneys were sectioned for histological study.   The kidney weights were determined in order to establish whether the diet affected them in the same manner as it did the development of renal lesions.   The adrenals were weighed because adrenalectomy inhibits the nephrosclerotic activity of L.A.P. (9) and because it has been claimed by some and refuted by others that the proportion of protein in the diet influences their weight (4, 18, 21, 50, 55).   The incidence and severity of nephrosclerosis was determined microscopically employing a scale ranging from 0, to indicate no lesions, to +++, to represent maximal damage.

* A commercial animal food prepared by Ralston Purina Co. Ltd., Montreal, Que.

** A commercial infant food prepared by Mead Johnson and Company of Canada, Limited, Belleville, Ont.

*** L.A.P. is prepared as follows: whole cattle pituitaries are removed from the skulls at the abattoir and immediately frozen in dry ice. At the laboratory, the glands are thawed, freed of posterior lobe and connective tissue, minced, and refrozen in bulbs. The bulbs are dehydrated in a high vacuum system containing a trap cooled with dry ice. The dry powder obtained suspends easily in water or 10% alcohol and passes through a No. 20 hypodermic needle.

Results: In Table I, the data obtained in Expt. 1 are summarized. Kidney weights are expressed in terms of mgm. per 100 cm.^{2} of body surface area. The incidence and severity of renal lesions are shown as percentages of the maximal ever observed.

TABLE I

INFLUENCE OF DIET ON THE NEPHROSCLEROTIC ACTIVITY OF L.A.P.

(Averages and standard errors)

Group	Treatment		No. of rats	Final body weight, gm.	Kidney			Adrenal, mgm.
	Dietary	Hormonal			Mgm. per 100 cm.^{2} body surface area	Nephrosclerosis		
						Incidence	Severity	
1	Purina	—	10	104 ± 5	418 ± 7	0	0	27 ± 2
2	50% Purina, 50% starch	—	9	93 ± 3	319 ± 10	0	0	25 ± 2
3	Purina	L.A.P.	8	131 ± 6	686 ± 32	88	38	64 ± 2
4	50% Purina, 50% starch	L.A.P.	5	140 ± 7	425 ± 6	0	0	41 ± 2

First, it should be pointed out that the 50% Purina diet was not markedly deficient in food essentials since the body growth rates of treated or untreated rats consuming this diet did not differ significantly from those of rats eating 100% Purina. No significant difference was seen in the adrenal weights of untreated rats consuming the two diets. However, the relative kidney weights of animals given 100% Purina (Group 1) were much greater than those of rats given 50% Purina (Group 2). No renal lesions were observed in any of these rats. With L.A.P. treatment (Groups 3 and 4), adrenal and kidney weights were increased in both groups, but much more in the group fed Purina only. Nephrosclerosis occurred in seven of the eight surviving animals given the 100% Purina diet, and in none of those eating the Purina–starch mixture.

These observations show that the diet can profoundly influence the development of the kidney lesions induced by hormone treatment. It is not clear, however, to what dietary factor the observed effects were due, since adding starch to the Purina not only increases the carbohydrate and decreases the protein content of the diet, but also decreases the proportion of fat and vitamins. It was decided to continue this study using simple synthetic diets whose constituents could be so varied that only two food factors would be changed at one time.

NEPHROSCLEROTIC ACTIVITY OF L.A.P. IN RATS ON TWO BASIC SYNTHETIC DIETS. THE COMPOSITION OF THE TWO BASIC SYNTHETIC DIETS

The constituents of the two basic synthetic diets and their proportions are given in Table II. The proportion of protein to carbohydrate in Diet 2 was chosen to coincide with that reported for Pablum, while the proportion in Diet 1, was made somewhat greater than that reported for Purina.

TABLE II

Composition of two basic synthetic diets

	Diet No.	
	1	2

Composition, %

Cornstarch	54	69
Casein*	30	15
Fat**	1	1
Cod liver oil ***	1	1
Bulk†	1	1
Sodium chloride ††	4	4
Mineral mixture‡	4	4
Water †††	5	5

Supplements, mgm. per 100 gm. of diets

Thiamine hydrochloride‡‡	0.4
Riboflavin‡‡	0.4
Pyridoxin‡‡	0.4
Calcium pantothenate‡‡	2.0
Nicotinic acid‡‡	1.0
α-Tocopherol acetate‡‡	About 10 mgm. per rat once weekly introduced by dropper into mouth

    * *Not vitamin-free. It was considered that this would supply the choline requirement.*

   ** *Crisco, Domestic Shortening, or Primex.*

  *** *Ingram and Bell.*

   † *Agar agar, acacia, or "Cellu-flour". When agar became unavailable we used acacia until Cellu-flour could be obtained. No difference was seen between them.*

  †† *This was added to the diet to sensitize the rats to the nephrosclerotic activity of L.A.P. instead of giving it in the drinking water.*

 ††† *The starch and casein in the first lot of diets was found to contain an amount of water corresponding to 5% of the diet. On the dry weight basis these diets contain 31.6 and 15.8% protein, respectively. Subsequently the water content of the starch and casein was determined and the amount weighed out calculated to give 54% or 69% starch and 30% or 15% casein by dry weight.*

  ‡ *Slightly modified mixture recommended by Steenbock and Nelson (52).*

NaCl	*23.4*	*gm.*
MgSO₄ . 7H₂O	*24.6*	*gm.*
Na₂HPO₄	*14.2*	*gm.*
K₂HPO₄	*69.6*	*gm.*
CaHPO₄ . 2H₂O	*69.8*	*gm.*
Ca-lactate . 5H₂O	*15.4*	*gm.*
Fe-citrate	*1.2*	*gm.*
KI	*0.16 gm.*	

 ‡‡ *Kindly supplied by Hoffman-LaRoche.*

It will be noted that inositol and liver extract are not included in these diets. The effect of adding these has not been investigated, but at the time it was felt that in such short term experiments as those to be described, they

would not be essential. Similarly, a simple salt mixture was employed in preference to a more complete one, since in three weeks, it was unlikely that trace elements would play any role.

THE ADEQUACY OF THE TWO BASIC SYNTHETIC DIETS

The adequacy of these diets for growth and maintenance has been tested in four different experiments. The data obtained are summarized in Table III.

TABLE III

GROWTH RATES AND ORGAN WEIGHTS WITH SYNTHETIC DIETS

(Averages and standard errors)

Experiment No.	Group No.	Diet No.	No. of rats	Body weight		Organ weights	
				Initial	Final	Adrenal, mgm.	Kidney, mgm. per 100 sq. cm. body surface
2 (W)	1	2	6	46	82 ± 4	19 ± 1	523 ± 9
	2	Pablum	6	46	84 ± 4	17 ± 1	467 ± 12
3 (BW)	3	2	10	50	98 ± 2	13 ± 2	379 ± 13
	4	1	10	50	113 ± 3	15 ± 2	454 ± 14
4 (W)	5	2A	6	50	106 ± 3	22 ± 1	427 ± 9
	6	1A	6	50	139 ± 5	35 ± 2	595 ± 17
5 (BW)	7	2A	8	122	251 ± 12	33 ± 2	491 ± 11
	8	1A	8	125	259 ± 21	30 ± 1	525 ± 19

The means given for Expts. 3 and 4 are also the untreated control values for the treated animals in the experiments to be described below. Different strains of rats were employed in different tests. The strain used is indicated by letters placed below the experiment number: *W*, for white (albino), and *BW*, for black-and-white or piebald. This factor is of importance when comparisons are made between experiments. Both strains respond to the same stimuli although average organ weights differ somewhat.

In *Expt. 2*, the body growth rate and organ weights of immature male rats eating Diet 2 for 21 days were compared with those of similar rats consuming Pablum. No difference in growth rate was seen. Of the organs weighed, only the relative kidney weights were significantly different. The cause of this is unknown; it might be due to a difference in the type of protein eaten.

In *Expt. 3*, a comparison was made of the body growth rates and organ weights of immature castrate rats partially nephrectomized and fed Diets 1 and 2 for four weeks. The growth rate of those fed Diet 2 was slightly less

than that of the rats eating Diet 1. No difference in organ weights was noted except in relative kidney weights. As would be expected, those animals consuming the low protein and high carbohydrate diet had the smaller kidneys.

Both these groups were in excellent condition, but in L.A.P. treated rats, Diet 2, unlike Purina, Pablum, or Diet 1, was quite inadequate for maintenance and rapid growth. Calculation suggested that the daily vitamin B intake was probably just adequate under normal conditions, but not after stimulation of metabolism with pituitary hormones. In experiments performed after this time, the vitamin B content of both diets was doubled. In addition, 100 mgm. of choline chloride was added per 100 gm. of the two diets. This markedly improved the condition of treated rats consuming the low protein, high carbohydrate diet, but had no apparent effect on that of the animals consuming Diet 1.

In *Expt. 4*, a comparison was made of the growth rates and organ weights of two groups of six immature, castrate rats, partially nephrectomized and eating these two diets so supplemented (Diets 2A and 1A, Groups 5 and 6). The additional vitamins markedly improved the growth rate. As might be expected, the animals on the low protein diet grew less rapidly, perhaps because their methionine intake was slightly below that considered essential for rapid growth (41). The adrenal and relative kidney weights of rats on Diet 1A were significantly larger than those of rats consuming Diet 2A.

Expt. 5 showed that with somewhat older rats, these two diets (Diets 2A and 1A, Groups 7 and 8), sufficed to maintain normal growth and good health for more than 11 weeks. The absence of any difference in growth rate further indicated that the inhibition noted in very young rats could be due to lack of methionine. As before, only the kidney weights differed significantly in the two groups.

It appears that the two basic synthetic diets were adequate for the normal growth of rats weighing 120 gm. or more; rats weighing 40 to 60 gm. grew less well on Diet 2 than on Diet 1 or Pablum.

The Nephrosclerotic Activity of L.A.P. in Rats Consuming Synthetic Diets

The nephrosclerotic activity of L.A.P. in rats kept on the above-mentioned diets was determined employing a previously described assay method (16). Male rats weighing 40 to 60 gm. were castrated, partially nephrectomized, and subcutaneously injected with L.A.P. for 21 days commencing one day postoperatively. In *Expt. 6*, the animals received 25 mgm. and in the rest, 20 mgm. daily, in two 0.3 cc. portions of a 10% alcoholic suspension. The average amount of dry food and water consumed by each rat was determined for the last five days in Expts. 6 to 9. As before, the fixed adrenals and kidneys were weighed, and the incidence and severity of nephrosclerosis were diagnosed histologically. Results of Expts. 6 to 9 are tabulated in Table IV

TABLE IV

The Effect of the Proportion of Protein in the Diet Consumed on the Nephrosclerotic Activity of L.A.P.

(Averages and standard errors)

Experiment No.	Group No.	Diet No.*	Diet Modification	No. rats	Final body weight, gm.	Nephrosclerosis Incidence	Nephrosclerosis Severity	Organ weights Adrenal, mgm.	Organ weights Kidneys, mgm./100 cm.² body surface	Food intake, gm./rat/day	Water intake, cc./rat/day
6 (BW)	1	2	Casein 50% Starch 34%	4	122 ± 7	0	0	48 ± 4	516 ± 17	10.7 ± 0.6	37 ± 2
	2	1		10	163 ± 5	90	47	71 ± 5	858 ± 50	9.0 ± 0.4	49 ± 4
	3	1		8	164 ± 10	100	61	81 ± 7	1107 ± 43	9.2 ± 0.6	99 ± 9
	4	2	Double B-vitamins Choline added	9	115 ± 5	0	0	53 ± 4	534 ± 15	7.2 ± 0.5	32 ± 2
	5	2		8	132 ± 5	0	0	49 ± 2	536 ± 20	9.3 ± 0.3	38 ± 2
7 (BW)	1	2	Double B-vitamins** Choline added	5	113 ± 9	0	0	58 ± 2	650 ± 36	13 ± 0.7	28 ± 3
	2	2		10	122 ± 4	0	0	58 ± 3	540 ± 19	13 ± 0.7	33 ± 2
	3	1	Double B-vitamins Choline added	5	146 ± 13	100	93	78 ± 7	983 ± 62	17 ± 1.2	58 ± 1
	4	1		8	151 ± 5	100	93	80 ± 4	991 ± 48	16 ± 0.7	57 ± 2
8 (BW)	1	1A	Cornstarch replaced by wheat starch	4	151 ± 8	100	92	91 ± 5	968 ± 84	17 ± 1	67 ± 4
	2	2A		3	104 ± 10	0	0	46 ± 4	472 ± 33	13 ± 1	23 ± 2
	3	1A	Cornstarch replaced by wheat starch	7	172 ± 5	86	53	72 ± 3	763 ± 66	17 ± 1	38 ± 3
	4	2A	Fat 8.5% Cornstarch 54%	8	115 ± 7	0	0	41 ± 2	458 ± 23	11 ± 1	30 ± 2
9 (W)	1	2A	{Fat 8.5% Cornstarch 54%	10	140 ± 6	0	0	54 ± 3	564 ± 23	16 ± 1	39 ± 3
	2	2A		7	133 ± 3	0	0	57 ± 4	613 ± 39	15 ± 1	38 ± 3
	3	1A	{Fat 8.5% Cornstarch 39%	9	148 ± 5	90	74	64 ± 3	1101 ± 74	15 ± 1	52 ± 4
	4	1A		9	154 ± 8	100	80	78 ± 5	1101 ± 52	14 ± 1	59 ± 5

* See Table II.
** Subsequently designated by "A."

and of Expts. 10 and 11 in Table V. They are expressed in the same terms as were employed in Table I. All groups originally consisted of 10 rats; the number indicated in the table is the number surviving to autopsy. Average initial body weights are omitted since they ranged from 52 to 54 gm. throughout.

TABLE V

THE INFLUENCE OF THE TYPE OF PROTEIN IN THE DIET ON THE
NEPHROSCLEROTIC ACTIVITY OF L.A.P.

(Averages and standard errors)

Experi-ment No.	Group No.	Diet		No. rats	Final body weight, gm.	Nephrosclerosis		Organ weights	
		No.	Modification			In-cidence	Severity	Adrenal, mgm.	Kidneys, mgm./100 cm.² body surface
10	1	1A		6	178 ± 9	100	82	63 ± 4	1100 ± 74
(W)	2	1A	{Casein 15% {Gelatin 15%	5	121 ± 7	80	47	54 ± 1	963 ± 16
	3	1A	{Casein 15% {Zein 15%	9	152 ± 6	78	44	62 ± 4	796 ± 41
	4	1A	{Casein 15% {Egg albumen 15%	8	144 ± 14	100	83	68 ± 3	1122 ± 53
	5	1A	{Casein 15% {Gluten 15%	7	157 ± 5	100	71	65 ± 4	1079 ± 84
11	1	1A		9	169 ± 6	100	80	69 ± 4	1066 ± 50
(W)	2	1A	{Casein 15% {Gelatin 15%	10	135 ± 5	70	30	53 ± 2	906 ± 48
	3	1A	{Casein 15% {Zein 15%	10	154 ± 6	70	40	56 ± 2	649 ± 43
	4	1A	{Casein 15% {Egg albumen 15%	6	137 ± 8	83	67	62 ± 5	1002 ± 83
	5	1A	{Casein 15% {Lactalbumin 15%	9	151 ± 5	66	54	59 ± 2	789 ± 52
	6	1A	{Casein 0% {Lactalbumin 30%	9	150 ± 7	56	26	56 ± 3	588 ± 16

The dietary factors studied included the effects of: (1) varying the ratio of protein to carbohydrate, (2) increasing the vitamin B content, (3) substituting wheat starch for cornstarch, (4) replacing a portion of the starch with an equicaloric amount of fat, (5) changing the source of protein.

(1) *Effect of Varying the Ratio of Protein to Carbohydrate and* (2) *of Increasing the Vitamin B Content of the Diet*

In *Expt. 6*, the nephrosclerotic effect of L.A.P. was assayed in rats consuming five different diets. Those in Group 1 were fed basic diet No. 2 (15% casein), and those in Group 2, basic diet No. 1 (30% casein). Rats in Group 3

consumed a diet in which starch was replaced by casein to make the regimen contain 50% casein. Animals in Group 4 were given Diet 2 supplemented with twice the amounts of B vitamins indicated in Table II. Those in Group 5 were fed Diet 2 supplemented with 100 mgm. of choline chloride per 100 gm. of diet. Nephrosclerosis developed only in those rats consuming diets containing 30% or more of casein. No kidney lesions were observed in rats eating any of the three diets containing 15% casein. The development of kidney lesions showed no relationship to the average food intake during the last five days, but was roughly proportional to the increase in body weight, adrenal size, relative kidney weight, and water intake. Increasing the concentration of B vitamins and choline decreased the mortality and greatly improved the condition of rats fed the low protein diet.

In *Expt.* 7, we further investigated the effect of vitamin B and choline chloride supplements on survival and the development of kidney damage. Four groups were employed, those in the first were given Diet 2; the second, Diet 2 modified (Diet 2A); the third, Diet 1; and the fourth, Diet 1 similarly modified (Diet 1A). Survival was again improved by the extra addition of these vitamins. Consequently they were added in all subsequent experiments. The addition of these vitamins also decreased the kidney weight of animals eating the low protein diet, but had no significant effect on adrenal enlargement or the development of kidney lesions. Again a rapid growth rate, large adrenals, large kidneys, and greatly increased water intake were associated with the occurrence of nephrosclerosis. In this experiment the rats consuming the low protein, high carbohydrate diet ate less in the last five days, than those given the high protein, low carbohydrate regimen, hence their salt intake was not the same. However, the difference involved could not be responsible for the difference in their sensitivity to the development of nephrosclerosis, since in Expt. 9 the difference in food intake was not manifest while that in the occurrence of kidney lesions was.

(3) *Effect of Substituting Wheat Starch, or* (4) *Fat, for Cornstarch*

It was possible that the nephrosclerosis preventing effect of the 69% cornstarch diet was due specifically to this starch. To investigate this, in *Expt.* 8, the whole of the cornstarch was substituted by wheat starch. To determine whether fat was also effective, a portion of starch in Diet 2A was replaced with an equicaloric amount of vegetable fat (Crisco) so that the diet contained the same proportion of starch as the 30% casein basic diet (Diet 1A). The difference in the weight of starch removed and fat added was made up by adding bulk (agar agar).

Nephrosclerosis occurred in rats eating the 30% casein, 54% wheat starch diet (Group 3) just as it did in those consuming the 30% casein, 54% cornstarch regimen (Group 1). No kidney lesions were seen in rats eating either the 15% casein, 69% wheat starch diet (Group 2), or the 15% casein, 54%

cornstarch, and 8.5% fat regimen (Group 4). Although survival was low in the first two groups owing to intercurrent infections, the results were so conclusive that it was decided not to repeat them.

However, the ability of fat to effect the same protective action as carbohydrate in the low casein diet was confirmed in *Expt. 9*. No kidney lesions appeared in rats eating food composed either of 15% casein, 69% starch, and 2% fat; or of 15% casein, 54% starch, and 8.5% fat. The development of severe damage likewise failed to be influenced by substituting fat for a similar amount of starch in the diet containing 30% casein and 54% starch.

In both these experiments, as in the previous two, the development of nephrosclerosis was associated with a rapid growth rate, markedly enlarged adrenals and kidneys, and a greatly increased water intake.

Thus, since wheat starch, vegetable fat, and probably other carbohydrates and fats can replace cornstarch, the kidney damaging effect of the 30% casein diet must have been due to the presence of protein and not to the lack of carbohydrate.

(5) Effect of Substituting Various Proteins for Casein

In Expts. 2 to 9, casein was used as the source of protein. To determine whether all proteins are equally toxic we examined the effect of substituting half of the casein in the 30% casein diet with the following protein preparations: gelatin, lactalbumin, zein, egg albumen, and gluten. Table V summarizes the results of Expt. 10, in which gelatin, zein, egg albumen, and gluten were used. It will be seen that the incidence and severity of kidney lesions differed greatly in the various groups. These differences are larger than could be expected on the basis of chance since the standard error of the incidence of the response in the four groups of rats consuming Diet 1A, unmodified, in Expts. 8 to 11 is 3 and that of the severity is 4. As in previous experiments the severity of kidney damage is correlated with the increase in adrenal and kidney weights.

Expt. 11 was essentially a repetition of the previous one with the addition of Groups 5 and 6 in which lactalbumin replaced respectively half or the total casein. From the results shown in Table V, it is seen that there was remarkable agreement with the responses obtained in the previous experiment, in respect to the incidence and severity of kidney lesions, growth rate, and the increase in adrenal and kidney weights.

These experiments show, therefore, that in rats injected with crude anterior-pituitary, the consumption of casein, egg albumen, and wheat gluten is associated with the development of greater kidney damage than is seen when lactalbumin, zein, and gelatin are eaten. We are now investigating whether the difference between these proteins is due to their different amino acid composition.

Discussion

PREVIOUS OBSERVATIONS

Diet and Chronic Nephritis

The influence of the protein content of the diet on the development of nephritis, nephrosclerosis, and malignant hypertension, has long been a subject of controversy. Workers who obtained kidney damage by giving protein-rich diets considered the lesions to be similar to those seen in chronic nephritis (13, 30, 33, 36, 43, 57). Workers who did not obtain lesions after prolonged treatment, claimed inadequate or unnatural diets were responsible for the positive results (2, 20, 35).

Older animals were found more responsive than young (19, 27, 51) and chronic nephritis occurs spontaneously in old rats and in rabbits of any age, hence it was suggested that the incidence of lesions obtained in the earlier experiments was not significant (35). More recently, partial nephrectomy (19, 27, 7), the use of liver as the source of protein, and treatment with thyroxin have reduced the required duration to three or four months (6). The lesions obtained in this shorter period were very similar to those seen normally in old rats.

The ability of various amino acids to reproduce the kidney-damaging effect of proteins has been investigated by several authors. Although tubular damage was noted after injecting or feeding a number of amino acids (12, 17, 22, 28, 32, 53), glomerular or arteriolar injury was mentioned only by Newburgh and Marsh (32) who reported prolonged albuminuria with the toxic amino acids, and Hueper and Martin (17), who observed hyalinization of the media of arterioles with tyrosine. Marked kidney enlargement followed the addition of glutamic acid to the diet but no specific lesions were noted (23). A probable metabolite of glutamic acid, glutaric acid, has been reported to be both toxic and nontoxic (39, 40, 58). Rose (40) obtained tubular nephritis with and without glomerular involvement, when the sodium salt of this acid was injected. In no case, however, did the damage approximate that seen with long protein feeding. Thus the effect of a high protein intake is probably not due merely to the absorption of large amounts of any one amino acid. It could be due, however, to an imbalance between the supply of certain amino acids and the rate of their utilization or destruction. Supporting such a suggestion of an indirect effect is the fact that the administration of certain amino acids or high protein diets is known to influence vitamin requirements (3, 6, 8, 11, 26, 29, 37, 38, 42, 44, 56).

Diet and Renal Hypertrophy

The effect of dietary protein on kidney enlargement has also been a subject of controversy. Those authors who observed kidney lesions described marked kidney enlargement. A similar renal hypertrophy was also seen by workers who found no lesions that they considered characteristic of chronic nephritis

(2, 34, 35). Since excessive diuresis, tubular dilatation, and damage were noted, it is possible that nephrosclerosis would have developed after a longer period of treatment. In short term experiments, MacKay *et al.* (25) noted that the amount of enlargement very closely paralleled the casein intake. Subsequently it was found (24) that the hypertrophy is greatly lessened, but not entirely prevented, if the same yeast extract: casein ratio is maintained in the low and high protein diets.

Diet and Adrenal Hypertrophy

Significant adrenal enlargement was noted in animals fed protein-rich diets by Tepperman *et al.* (55), but not by Ingle *et al.* (18) or others (4, 21). It is possible that the vitamin intake might play a role in enlargement as it certainly does in the maintenance of normal adrenal structure (29, 54).

Chronic Nephritis and the Growth Rate

It has been remarked repeatedly that chronic nephritis occurs more readily in otherwise healthy and well-developed animals than in the ill-fed. For example, Newburgh and Curtis (30) pointed out that "the diets that caused the most injury also permitted the best growth". Similarly, Goldblatt *et al.* (14) stated that a high meat diet induced hypertension only in growing animals. Clinically, when nephritis follows an infection, it tends to occur during convalescence. In pregnancy, it is often seen when the fetus is gaining weight most rapidly. The role of protein in the induction of chronic nephritis has usually been attributed to improper catabolism, but it should be kept in mind that it might be correlated with an anabolic imbalance.

Chronic Nephritis and Caloric Intake

It is also possible that the damage seen is associated with a high caloric intake rather than rapid growth or repair, since in all the above-cited situations, excessive amounts of food are consumed. In support of this possibility is the sensitizing effect of thyroxin. Animals treated with this hormone have greatly increased appetites but may gain or lose weight depending on the adequacy of the diet (10). The finding of Saxton and Kimball (43) that restriction of the food intake reduces the incidence of spontaneous "chronic nephrosis" is also pertinent.

Present Observations

Relation between Chronic Nephritis and Nephrosclerosis

The kidney lesions that we obtained in rats treated with anterior-pituitary preparations are very similar to those shown in the excellent illustrations of Jackson and Moore (19). As with lesions of purely dietary origin, their development is promoted by partial nephrectomy (16), thyroxin (47), and, as shown here, by the feeding of a moderately high protein diet. The effect of a high sodium chloride intake, which is also sensitizing in L.A.P.-treated

rats (16), has not been adequately investigated in respect to the production of purely dietary chronic nephritis. When salt comprised 18 to 25% of the diet, no sensitizing effect was seen (35), but it is probable that the test rats refused to eat adequate amounts of such a diet.

The production of a high incidence and severity of kidney lesions in one-month-old rats treated for 21 days, represents a considerable acceleration of a process that could be expected to run its course in three to five years in a normal rat, about one and one-half years in a partially nephrectomized rat on a protein-rich diet, and three to four months in a partially nephrectomized rat, kept on a similar diet and given thyroid.

Dietary Protein: Carbohydrate Ratio and Nephrosclerosis

There can be no doubt that the production of these kidney lesions with L.A.P. treatment is partly dependent upon the protein intake. Various protein preparations caused different degrees of kidney damage in agreement with the findings of earlier workers who used purely dietary measures to produce renal lesions (13, 30, 31, 43). The feeding of carbohydrate or fat instead of protein protected rats against the nephrosclerosis-producing effect of L.A.P. The growth rate and food intake were often less with the non-damaging synthetic diets: this may have played some role but could not have been the sole cause of the difference in toxicity. As shown in Table I, the growth rate of the animals given the two diets did not differ significantly, yet only those eating Purina developed lesions. Moreover, as shown in Table V, synthetic diets containing different protein preparations permitted identical growth rates with very different degrees of kidney damage.

Vitamin Supply and Nephrosclerosis

The nephrosclerosis is not prevented by a normal supply of vitamins. The same concentration of vitamins was present in the diets of the rats that had pathologic kidneys and those that did not. Further work with vitamins of the B complex is being done, however, in view of the repeated claims that the vitamin intake markedly influences the development of chronic nephritis of purely dietary origin.

Urine pH and Nephrosclerosis

The production of nephrosclerosis in these experiments was accompanied by no gross change in the pH of bladder urine*. The urine pH of untreated castrates kept on Diets 1A and 2A was 6.2 ± 0.1 and 6.2 ± 0.1, respectively (50 determinations each), and that of L.A.P.-treated castrates, 5.9 ± 0.1 and 5.8 ± 0.1, respectively (100 determinations each). It appears that L.A.P. caused no significant decrease in urine pH.

* *Measured visually by expressing bladder urine on two or three ranges of "Accutint" pH papers.*

Urine Albumin and Nephrosclerosis

The urine was tested for albumin in a number of the experiments*. At the end of an experiment, the albumin concentration in the urine of rats kept on Diet 2, modified and unmodified, was the same as in stock rats, while all those receiving Diets 1 or 1A had a moderate to excessive albuminuria. In the latter groups, the increased output began after the fifth day, but was approximately maximal by the 10th day. The amount lost varied somewhat from day to day and with the protein preparation fed. For example, animals consuming the mildly toxic 30% lactalbumin diet averaged on three occasions in the last 10 days an incidence and severity of 66 and 23% albuminuria as compared with 100 and 52% for rats given the highly toxic 30% casein regimen.

Renal Hypertrophy and Nephrosclerosis

In L.A.P.-treated rats the kidney enlargement was roughly proportional to the degree of nephrosclerosis produced. No consistent renal enlargement was seen in groups that developed no lesions; the differences in kidney weight are so small that it is necessary to have untreated control animals with each experiment to demonstrate the purely renotrophic effect of anterior-pituitary preparations (15).

Diet and Adrenal Hypertrophy

The adrenal enlargement also roughly paralleled the development of the kidney lesions, but a very marked increase in adrenal weight occurred also in L.A.P.-treated rats that developed no renal lesions. Occasionally, partial or nearly complete cortical necrosis was seen with excessive adrenal enlargement in L.A.P.-treated animals on Diet 1 modified or unmodified. Adrenal necrosis was not seen in treated animals consuming Diet 2 modified or unmodified.

Acknowledgments

* *Determined in expressed urine by adding 0.3 cc. of Esbach's picric and citric acid reagent to 0.3 cc. of urine in a conical 10 cc. centrifuge tube, centrifuging 10 min. at 1500 r.p.m. The incidence and severity of albuminuria, read using a scale of 0 to $++++$, was then expressed as a ᵗ⁻⁻⁻ᵗᵃᵍᵉ ᵒf ᵗᵏᵉ ᵐᵃᵡⁱᵐᵃⁱ ᵖᵒˢˢⁱᵇⁱᵉ.*

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FLUORESCENCE MICROSCOPE EXAMINATION OF TRYPANOSOMES IN BLOOD[1]

BY S. STRUGGER[2]

Abstract

With acridinorange it is possible to stain selectively trypanosomes in blood intra-vitam. In subsequent examination by means of a fluorescence microscope only leucocytes and trypanosomes are visible (as shining bright green bodies), while the erythrocytes are not visible. Living and dead trypanosomes, too, may be distinguished in this way.

With the fluorescent dye auramine, trypanosomes in blood may be selectively stained (showing up in golden fluorescence) so that diagnosis for trypanosomes is very greatly facilitated. Even in difficult cases a decision on the presence of trypanosomes in blood is possible.

The staining of blood smears by methods normally used and microscopic examination makes it possible to find trypanosomes in blood easily only if they are present in a sufficient number. But if there are only a few trypanosomes, they may often be overlooked because of the immense mass of deeply colored erythrocytes. In such cases microscopic examination for trypanosomes is usually so difficult that, in practice, it cannot be carried out.

The following paper describes two fluorescence microscope methods with the aid of which microscopic diagnosis for trypanosomes may be so facilitated —even in difficult cases—that even single trypanosomes can be found quickly and accurately. Jancsó (1932 (2)) succeeded in making trypanosomes visible under the fluorescence microscope by the use of trypaflavine. Hirt (1939 (1)) described an intravital staining of trypanosomes in blood of animals that were injected with trypaflavine. Both of these methods have been used mainly for chemotherapeutical purposes.

When Strugger (1940 (3)) discovered that acridinorange (3,6-tetramethyl-diaminoacridin) is a vital dye that will stain the protein structures of living protoplasm, he succeeded in finding a method of selective vital staining of living trypanosomes in blood. As fluorochrome a solution of acridinorange 1:10,000—made with 0.85% sodium chloride—is used. A drop of freshly

taken blood is mixed with a drop of this acridinorange solution on a slide and covered with a cover slip. The examination is carried out with a blue light fluorescence microscope.

Anyone may easily construct such an instrument. The source of light used is a carbon-arc lamp with attached convex lens by which a bundle of parallel rays is produced. The light is filtered by a cuvette (2½ cm. thick) filled with a solution of saturated copper oxide ammonia so that only blue light reaches the plane mirror of the microscope. The microscope is of the ordinary type but has a filter inserted over the ocular. The filter is an orange glass that absorbs the blue light quantitatively but allows green, yellow, and red light to pass through quite unchanged. To focus the fluorescence microscope, a slide, on which is placed pulverized anthracene in liquid paraffin, is used.

With ordinary magnification, the observation of a blood smear stained with acridinorange gives the following image. The erythrocytes are non-fluorescent and scarcely visible. Sporadically situated leucocytes are vitally stained with acridinorange. Their nuclei and protoplasm have a strong green fluorescence. (It may be noted here that a very accurate counting of the leucocytes may be carried out in this way.) The trypanosomes shine with bright, light green fluorescence, do not lose their motility, and are therefore quite easy to find under low magnification. Cytological examination of the living trypanosomes is possible under an oil immersion lens. The cytoplasm fluoresces a diffuse green and the cilia, too, are stained. The nucleus is visible in bright yellow-green fluorescence and the blepharoplast may be very clearly observed.

This method of staining trypanosomes vitally with acridinorange may be used for diagnostic purposes in difficult cases but, because of the different coloration of living and dead protoplasm discovered by Strugger (1940 (3), 1948 (4)), it should be most useful for chemotherapeutical research.

To facilitate microscopic examination for trypanosomes, a simple method has been elaborated that has the same optical advantages as the former and is applicable on dried blood smears.

A smear of blood is made and, after drying, fixed for two to three minutes in methyl alcohol. After a short washing it is stained for four minutes in a solution of auramine (1 part of auramine dissolved in 1000 parts of distilled water, with 5% liquid phenol added after that). Then it is washed in distilled water (one to two minutes) and dried in air. The durability of this stain is satisfactory if the slide is kept in darkness.

Examination of the preparation with the blue light fluorescence microscope is first made at a low magnification (100 to 200×). To find single trypano-somes in difficult diagnostic cases it is well to search first at low magnification and then at higher magnification. The following image is obtained. The background is black. The erythrocytes shine slightly, as dark green circles.

PLATE I

FIG. 1. *Fluorescence microscope photograph of Trypanosoma brucei in blood of a guinea pig, stained with auramine. The erythrocytes are visible as slightly green fluorescent circles. The leucocytes cannot be distinguished. Only the trypanosomes are shining in bright golden fluorescence.*

The leucocytes cannot be distinguished. Only the trypanosomes are marked by a bright golden fluorescence. Their shape and their inner structure may be excellently observed. The contrast is considerably improved if a drop of liquid paraffin and a cover slip are put on the stained smear.

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THE POTENTIATION OF INSULIN BY SULPHONES[1]

By A. Bruce Macallum[2]

Abstract

Sulphones in trace quantities combined with diet rich in fresh vegetables produce an increased sensitivity to insulin, both in rate of fall of blood sugar levels and maintenance of hypoglycaemia. The sensitivity is not contingent on the presence of sulphone compounds and may persist for several days after these compounds have been detoxicated or eliminated.

The results hereinafter described were obtained from experiments designed to test Keilin's theory that sulphonamide reacted with the zinc in carbonic anhydrase, thus rendering it inactive. If this hypothesis is applied to zinc insulin, it would seem that simultaneous administration of insulin and sulphonamide in rabbits should tend to produce a partial restriction of the hypoglycaemia. This assumption has some apparent support as Greisheimer (3) has shown that sulphonamides produce hyperglycaemia in rats. A preliminary trial with insulin and sulphonamide resulted not in inhibition but in enhancement of the insulin hypoglycaemia. Accordingly the study was reoriented to investigate the potentiating effect of sulphones, as a class, on insulin. This communication is an expanded account of the experiments referred to in an extremely brief preliminary note (7), together with later developments, and is in the nature of an interim report as the problem, which was temporarily suspended owing to wartime controls and postwar teaching load, has been resumed.

Methods

Rabbits were used; 170 single experiments were carried out on 70 animals. Since 1930 the writer has been engaged in studies on insulin synergism and during this period data were accumulated from the behavior of normal animals on insulin in amounts ranging from 0.1 to 0.5 units of insulin per kgm. These are shown in Fig. 2, curve No. 1 and are used as a basis of comparison with insulin in conjunction with sulphones.

The animals were kept in the animal house two to three weeks before use and fed on a standard diet. They were starved 24 hr. before use.

In the first series saturated solutions of sulphanilamide were used. Subsequently, in order to relate the molar concentration of sulphone to the unit value of insulin 1 ml. of a 0.01 M sulphone solution was used in conjunction with one unit of insulin. In the case of less soluble preparations more dilute solutions were used but the volume of the dose increased to keep the amount of sulphone in relation to the amount of insulin constant. The sulphone

solutions were injected hypodermically into the side of the animal opposite to the site of insulin administration in order to avoid formation of possible insulin-sulphone complexes.

Blood sugars were determined prior to 1938 by the Folin-Wu method and subsequently by the Shaffer-Hartmann-Somogyi method. All curves of insulin action were plotted using the fasting level as the base and the actual fall from and the return to the initial values expressed as the hypoglycaemia. This eliminated differences in values due to the two methods and makes the results comparable.

The values are expressed in milligram hours. The unit value of insulin is determined by two factors (*a*) the fall in blood sugar values, (*b*) the duration of the hypoglycaemia. A drop of 1 cm. on the ordinate of the graph paper is 10 mgm. % (10 mgm. glucose in 100 cc. blood) and 1 cm. on the abscissa represents one hour, so that 1 cm.^{2} = 10 mgm. hr. Integrating the area of the curve (the shaded areas in Fig. 1) with a metric planimeter and multiplying the total by 10 gives the total mgm. hr. of the hypoglycaemia. These values were used for determining the potentiating effect of sulphones against the same values of insulin alone.

Results

Sulphanilamide and sulphathiazole were the first to be used. These displayed a marked and spectacular increase in sensitivity to insulin. Subsequently the study was extended to include available sulpha drugs and simple and complex sulphones that were readily available. If a sulphone derivative showed no effect on two animals it was rated as negative. Where four out of five animals showed a marked and spectacular increment of the hypoglycaemia they were accepted as positive.
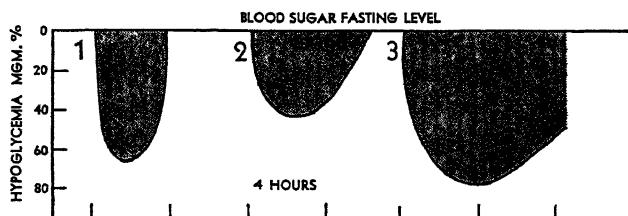
Fig. 1. *Curve No. 1: 0.1 units insulin, 1.5 ml. 0.01 M taurine solution per kgm.; No. 2: standard composite curve, 1.5 units insulin per kgm.; No. 3: 1.5 units insulin, 0.75 ml. 0.01 M saccharin per kgm.*

Typical examples of potentiation are shown in Fig. 1. Curve No. 2 is a composite, the data being obtained from a series of normal insulinized rabbits on 1.5 units per kgm. The points on the curve represent the arithmetical mean of decreases in blood sugar values. Curve No. 3 is the potentiating effect of 0.75 ml. 0.01 *M* saccharin on 1.5 units insulin per kgm. This experiment was terminated after eight hours, before the blood sugar had returned to the fasting level. Curve No. 1 is the effect of 1.5 ml. 0.01 *M*

taurine solution on 0.1 units insulin per kgm.　Here the insulin effect has been increased approximately fourfold.　Where the standard ratio of sulphone/insulin failed to give a complete result in the eight hour period it was found necessary to reduce either the amount of sulphone or insulin. This was employed when the effects were catastrophic, accompanied by a high mortality rate from convulsions when the standard ratios were used.
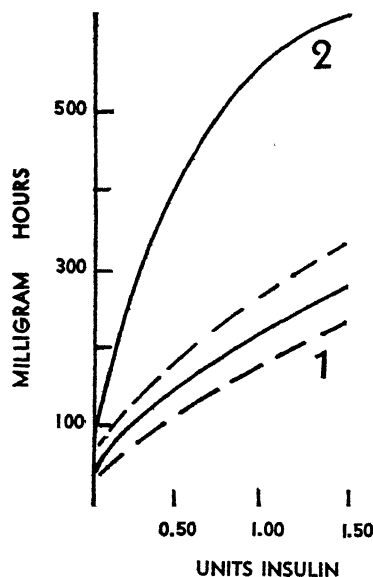
FIG. 2.　*Curve No. 1: standard normal curve of insulin—dotted lines indicate standard deviations from mean; No. 2: potentiating effect of 1.5 ml. 0.01 M taurine per kgm.*

Fig. 2 illustrates the sensitizing effect of taurine (1.5 ml. of 0.01 M per kgm.) on varying doses of insulin.　In the lower curve (No. 1), the solid line represents the effect of varying amounts of insulin in rabbits (the values on the curve being statistically corrected), the dotted lines indicating the standard deviations.　The upper solid line (No. 2) demonstrates the effect of the sensitizing action of taurine on the insulin.　The 1.5 unit value in this case was calculated from an incomplete experiment described previously, the blood sugar levels not returning to the base line in eight hours.　Projecting the curve back to the base line in this case gives a value that, if used, would make the curve register a much higher value for 1.5 units of insulin.

In all cases where definite increases in sensitivity to insulin were brought about by sulphones, the effect on insulin alone, subsequently administered, persisted in a gradually decreasing degree from 3 to 15 days, occasionally longer, after termination of sulphone administration.

A summary of the relative effects of the various members of the sulphone class investigated is shown in Table I where they are classified in their main chemical groupings.　In the case of simple sulphones the potentiation did

not appear until sulphamide was used and the activity increased in the subsequent members of this group, the maximum effect being attained with phthalyl tauramide. Tauramide hydrochloride (not shown in the table) was also tested and was about equal to taurine. In the case of sulpha drugs the potency was least with sulphanilamide, but increased in the succeeding members of this series, the last, No. 307, a disulphone under experimental trial, being the most effective in this group. In the benzene sulphonic derivatives, benzene sulphonic acid was questionable but the other members of this group were the most active of all of the sulphones investigated.

These observations were completed at the end of 1942, during a period when the animals were supplied from a single dealer who raised them on a specified diet for some weeks prior to delivery. Subsequently this source of supply ceased and the extension of wartime controls and the increased cost of feed made it necessary to use animals available regardless of source or dietary history and a commercial chow diet was substituted for that previously used. The commercial diet apparently did not differ from the previous standard diet in regard to its effect on sugar tolerance, behavior of the animal to insulin, and maintenance of general nutritional levels. However, animals on this regime displayed reduced potentiation when sulphones were used in conjunction with insulin and only sulphones that gave the maximum effect showed even moderate activity.
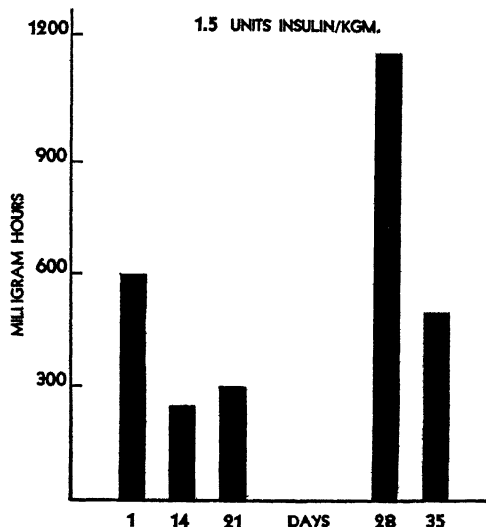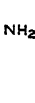
FIG. 3. *All animals get 1.5 units insulin per kgm. Day 1, added 1.5 ml. 0.01 M taurine per kgm. Days 1 to 21, commercial diet. Days 22 to 35, standard laboratory diet.*

Fig. 3 illustrates this point and was typical of every animal in this group on the commercial diet. Insulin was given periodically on the days indicated in the diagram. On the first day 1.5 ml. of 0.01 *M* taurine was given with the insulin, but was discontinued thereafter. For the first 21 days the animals

were on the commercial diet but on the 22nd day the standard diet used in the earlier series of experiments was used. The taurine effect on the first day was not so pronounced and the insulin effect on the 14th and 21st day was within the normal range. After seven days on the standard diet (the 28th

TABLE I

DEGREE OF POTENTIATION OF INSULIN ACTION BY VARIOUS SULPHONES

	Formula	Degree of potentiation
Simple sulphones		
Sulphamic acid		—
Ammonium sulphamate		?
Sulphamide		+ +
Taurine		+ + +
Phthalyl tauramide		+ + +
Benzene sulphonic acid derivatives		
Benzene sulphonic acid		?
Ethyl benzene sulphonate		+ + + +
Saccharin		+ + + +
Benzene sulphonamide		+ + + +
Sulphonamide drugs		
Sulphanilamide		+ +
Sulphathiazole		+ + +
Sulphaguanidine		+ + +
Sulphadiazine		+ + + +
Expt. No. 307		+ + + +

day) insulin was given with most spectacular results, which persisted even on the 35th day or longer. These had never occurred previously in many years of observation on insulinized animals. This experiment illustrates the persistence of the sensitizing effect of the sulphones, which was the result of the conjoint action of the sulphone and an unsuspected trace factor in the diets used prior to the change to the commercial diets. This factor was previously unsuspected and not manifested till the change in feeding took place. Every animal in this series displayed this latent effect of the sulphone and the dietary factor, some even more spectacularly than that shown in the illustration. The standard diet contained fresh cabbage, lettuce, carrots, hay, and oats—fresh vegetables being absent from the commercial diet, which was made up from mixtures of mill feeds. It is therefore apparent that the potentiating effect shown in Table I is due to the combined effect of the sulphone sensitization and some element in the fresh vegetables in the standard diet.

The crossover test usually applied in insulin standardization cannot be used without considerable modification in conjunction with sulphones. Owing to the persistence of the potentiating effect, crossing in 24 hr. intervals is out of consideration. At least two weeks must elapse before the normal insulin levels of action return and even then not all animals may be free of sulphone effect. Again a milder preparation must be used to avoid possible death from hypoglycaemic shock so that these conditions tend to render the crossover test less certain. Table II illustrates a modified crossover test, which, under the conditions referred to above, is about the best that can be expected

TABLE II

EXTENT OF HYPOGLYCAEMIA IN MILLIGRAM HOURS IN MODIFIED CROSSOVER TEST

	Group A	Group B
Day 1	1.5 Units insulin per kgm. 1.5 Ml. 0.01 M tauramide per kgm. 650 Mgm. hr.	1.5 Units insulin per kgm. 420 Mgm. hr.
Day 14	1.5 Units insulin per kgm. 440 Mgm. hr.	1.5 Units insulin per kgm. 1.5 Ml. 0.01 M tauramide per kgm. 560 Mgm. hr.

in view of the 14 day interval and the predictable activity of a sulphone of moderate sensitivity. While smaller amounts of insulin might have been used, subconvulsive levels of blood sugars were desirable to approximate more closely the actual testing conditions for determining unit values.

Discussion

The possibility that insulin forms a slowly diffusible complex with sulphones has been advanced and Sahyun and Heyn (6) found that insulin and brom-

camphor sulphonic acid, camphoric and camphoronic acids, when mixed under specified conditions, formed slowly acting depots with prolonged hypoglycaemic effect. The writer noted that sulphones and insulin mixtures in several cases formed precipitates or colloidal suspensions. To avoid the possibility of 'depots', insulin and sulphones were administered separately on opposite sides of the test animal. The writer's findings do not support the above view. The fall in blood sugar levels is extremely rapid and catastrophic and the sulphanilamide appears in the blood plasma in two hours and disappears about an hour afterwards, long before the blood sugar levels return to the initial fasting levels. Furthermore the sensitivity to insulin once established persists for days or weeks. Again the presence of a dietary factor in the potentiating mechanism together with these facts indicate that much more is involved than an insulin 'depot' and the effect is comparable to the action of synergists of pancreatic and duodenal origin (1, 4, 5).

Sulpha drugs in therapeutic doses do not depress blood sugar levels. Greisheimer *et al.* (3) found that in the range of 100 mgm. per kgm. the effect was hyperglycaemic. In the present investigation doses were of the order of 2 to 5 mgm. per kgm. Goldberg and Jefferies (2) used comparable doses in investigating the potentiating effect of sulphanilacetic acid. Some of the sulphones in these trace quantities showed slight hypoglycaemic action when used alone, but those most active in activating insulin had no effect on the blood sugar levels.

At this stage no discussion of the sensitizing mechanism is included owing to the presence of the uncontrolled variable—the trace factor in the green vegetables of the standard diet—whose activity was manifested in experiments of which the example in Fig. 3 is typical. Further investigations as to the nature and action of this component are in project.

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THE FORMATION OF ACETATE IN BRAIN TISSUE SUSPENSIONS[1]

By J. L. Webb[2] and K. A. C. Elliott[3]

Abstract

Observations have been made on methods for the determination of acetate and acyl phosphate in tissue suspensions. Previous work on the formation of acetate by respiring brain suspensions, especially in the presence of added pyruvate, has been confirmed. No evidence could be obtained that the substance formed is actually acetyl phosphate, which breaks down to yield acetic acid in the course of the estimation or enzymatically during the incubation of the tissue. The amount of acetate formed in brain suspensions is increased by the presence of malonate. It seems also to be increased by the addition of malate or α-keto-glutarate and by thiamine. Spontaneous breakdown of pyruvate could account for part of the acetate formed from pyruvate added to tissue. Fluoroacetic acid interferes with the determination of acetic acid. There was no evidence that it increases the formation of acetate by rat brain tissue suspensions. It does not affect the oxygen uptake of these suspensions with glucose or pyruvate as substrates.

Much work in recent years has indicated that some reactive two-carbon compound or radical is a key intermediate in the metabolism of carbohydrate and of fat and related substances via the Krebs cycle and in the synthesis of certain complex molecules. Such a two-carbon body is probably also the source of the acetyl group of acetylcholine, which appears to be an essential substance in the functional chemistry of nervous tissue.

Acetic acid is well known to be produced from alcohol, pyruvate, and other substances by various microorganisms. Among animal tissues, Krebs and Johnson (12) and Elliott et al. (6) found that, anaerobically, testis caused a dismutation of pyruvate whereby one molecule of pyruvate was reduced to lactate while another molecule of pyruvate was oxidized to acetate and carbon dioxide. The dismutation occurred to a considerably smaller extent in tissues other than testis.

Weil-Malherbe (22, 23) detected the formation of small amounts of acetate from pyruvate by minced ox brain, both anaerobically and aerobically. Aerobically, Long (16) found acetate production from pyruvate with pigeon brain mince and estimated that about 25% of the pyruvate used was converted to acetate. With rat brain suspensions, Elliott et al. (8), using accurate analytical procedures, found that about 14% of the pyruvate utilized aerobically was oxidized to acetate. These authors also found that small amounts of acetate were produced during the oxidative metabolism of glucose and lactate.

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Acetate is consumed by intact animals (see for example (3, 20)) and by various tissues *in vitro* (4, 6, 24). However, Elliott *et al.* (6, 8) found no sign of any utilization of acetate by rat brain tissue slices or suspensions. It seemed possible, therefore, that acetate is not a normal product of brain metabolism. Possibly a different substance that estimates as acetate in the analytical method is the actual metabolite formed. Such a substance might be acetyl phosphate, which is readily hydrolyzed to acetic acid and phosphate and which is an intermediate in the oxidation of pyruvate by certain bacteria (14). Or acetate formation may be an abnormal process occurring under *in vitro* conditions. In an interesting study of the metabolism of the malaria parasite, Speck *et al.* (21) found that acetate formation from pyruvate was greater with free parasites than in parasitized erythrocytes, possibly because the erythrocyte provides a more 'normal' environment, and was increased by high pyruvate concentrations. Additions of catalytic amounts of the dicarboxylic acids of the Krebs cycle slightly decreased the acetate production by free parasites, while malonate, which interferes with the cycle, increased the acetate formation. It was suggested that pyruvate, or a reactive two-carbon fragment formed from pyruvate, is normally taken up and oxidized via the Krebs cycle but under some conditions it may be transformed into acetate.

In the present study, isotonic brain suspensions, which have been shown to be comparable to brain slices in rate and type of metabolism (7) and lend themselves to accurate analytical studies, have been used. The observations of Elliott *et al.* (8) on acetate formation have been confirmed. No evidence that the substance actually formed is acetyl phosphate could be obtained. Acetate production was possibly slightly increased when the tissue was cytolyzed by homogenization in hypotonic medium, a condition that might perhaps be compared with that of the free malaria parasites mentioned above. Acetate formation was considerably increased in the presence of malonate but it was also somewhat increased when malate or α-keto-glutarate was added. An appreciable amount of acetate is formed from pyruvate under the experimental conditions in the absence of any tissue.

Bartlett and Barron (1) and Kalnitsky and Barron (11) have found that fluoroacetate inhibits the oxidation of acetate by kidney, liver, and muscle and by yeast and certain bacteria, and that in the presence of fluoroacetate the utilization of added pyruvate is diminished while acetate accumulates. They concluded that oxidation of pyruvate to acetate is of great importance in animal tissues. We have obtained no evidence that fluoroacetate interferes with the respiration of rat brain suspensions with either glucose or pyruvate as substrate, or that it increases the accumulation of acetate very markedly. Fluoroacetate interferes considerably with the determination of acetic acid.

In preparation for this work some observations have been made on the methods for acetate and acetyl phosphate determinations.

Methods

Suspensions, containing usually 150 mgm. tissue per ml., were prepared from whole rat brains by homogenization in calcium-free Ringer–0.017 M phosphate solution by means of the apparatus of Potter and Elvehjem (17). In some cases 0.11 M sodium phosphate, pH 7.3 to 7.5, was substituted for sodium chloride in the medium. Samples of the suspension, usually 3 ml., were pipetted into Barcroft manometer flasks and incubated for an hour with shaking at 38° C. Substrates, inhibitors, etc., or equivalent amounts of sodium chloride, dissolved in 0.3 ml. water, were introduced into the flasks before the suspension. The gas phase in the flasks was air. When oxygen uptake was to be measured, the center wells contained alkali-soaked filter paper rolls. At the end of the experimental period, the suspension from two flasks was pooled and samples were taken for acetic acid or acetyl phosphate determinations. A number of determinations were also made on suspensions deproteinized immediately after homogenization.

Acetic acid was determined by the method of Elliott *et al.* (8). The suspension was deproteinized with zinc sulphate and sodium hydroxide, and a 5 ml. sample of the filtrate, representing 1.7 ml. of tissue suspension or 250 mgm. of tissue, was run into the distillation flask, which contained 2 gm. of acid potassium phosphate. Steam distillation was carried on at atmospheric pressure until 50 ml. of distillate had collected. The distillate was rendered alkaline, evaporated down, and redistilled with 0.5 ml. of phosphoric acid. The second distillate was aerated with carbon dioxide-free air and titrated with freshly standardized carbon dioxide-free 0.005 to 0.008 N sodium hydroxide using bromthymol blue as indicator. The double distillation almost completely obviates interference by lactic and pyruvic acids. The apparatus at first used was copied, evidently imperfectly, from apparatus that was lent to one of us (K.A.C.E.) by Dr. W. C. Stadie and used in earlier work. It was found that variable and often very high blank titrations were obtained owing to traces of phosphate being carried into the distillate. This disturbance was mainly due to the collection of spray and condensed steam in the neck of the flask (A in Fig. 1) where it partially blocked the neck so that rapidly flowing steam could carry droplets of this collection further and into the condenser. Satisfactory results were obtained when new apparatus was constructed in which the neck was widened, the tube (B) to the Kjeldahl trap was widened, and a perforated bulb (C) was used for the steam inlet to allow smoother distillation. In Table I results of tests of the method are shown.

The method determines acid volatile with steam under the conditions used and is not specific for acetic acid. Acetate is the most probable substance that is formed in the tissue and fulfils these conditions but it is recognized that throughout this study the term "substance estimated as acetate" should be used instead of "acetate." All determinations were done in duplicate. But differences in individual figures in Tables II, IV, and V up to 2 μmoles per gram are within the limits of errors of the method and are probably not significant.

TABLE I

ACETIC ACID DETERMINATION. BLANKS AND RECOVERIES

The 'blanks' and 'samples' consisted of 5 ml. of solution containing 2 mgm. of sodium pyruvate and 2 mgm. *dl*-lactic acid and the amount of acetic acid shown

	Titration, ml. 0.005 N NaOH, average (range)	Equivalent acetic acid, mgm., average (range)	Recovery* %
Distilled water	0.16 (0.09-0.20)	0.05 (0.03-0.06)	
Complete distillation blanks	0.24 (0.15-0.38)	0.07 (0.05-0.11)	
Sample 0.27 mgm. acetic acid	1.15	0.34	100
" 0.35 " " "	1.38	0.41	97
" 0.47 " " "	1.80 (1.76-1.86)	0.54 (0.53-0.56)	98-102
" 0.71 " " "	2.58	0.77	98

Corrected for average distillation blank.
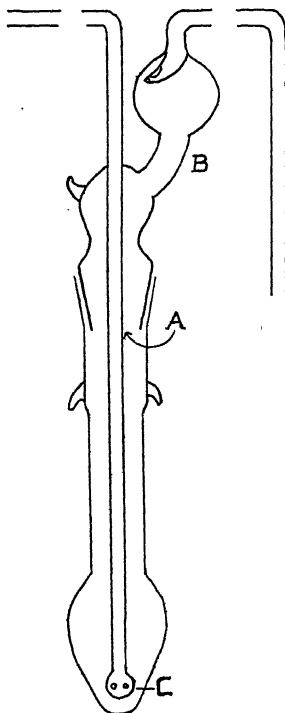
FIG. 1.   *Steam distillation flask for acetic acid determination.   The total height is 25 cm. The clearance at point A is at least 5 mm.*

Acetyl phosphate was determined by adaptations of the method of Lipmann and Tuttle (15), which consists in treating the sample with freshly neutralized excess of hydroxylamine hydrochloride solution, pH 6.4.   The hydroxylamine reacts with acyl phosphate to give the hydroxamic acid.   After deproteiniza-tion, the filtrate is treated with ferric chloride, which produces a brown-purple

color with the hydroxamic acid formed. The depth of color is measured with a photoelectric photometer. The color is compared with standards prepared from succinic anhydride, which gives the monohydroxamic acid. The color developed with ferric chloride in the standard corresponds to 80% of that given by an equivalent amount of the acetohydroxamic acid formed from acetyl phosphate.

In the original method, deproteinization was achieved with trichloracetic acid. With the tissue suspensions used here, it was found that cloudy filtrates were always obtained after the addition of even very high concentrations of trichloracetic acid, when high concentrations of hydroxylamine were present.* Perchloric acid was similarly unsatisfactory. Metaphosphoric acid gave clear filtrates but prevented the development of color with ferric chloride. In an alternative procedure described by Lipmann and Tuttle for use when there were present high concentrations of phosphate that depress the intensity of the color developed, a solution of zinc chloride and calcium chloride was added, followed by an amount of sodium hydroxide approximately equivalent to the zinc. However, we found that the strong buffering action of the hydroxylamine prevented the necessary formation of zinc hydroxide so that deproteinization was not achieved. In the method adopted here, sufficient relatively strong sodium hydroxide was added to overcome the buffer action of the hydroxylamine. This produced a copious precipitate, with deproteinization, and a clear filtrate was obtained. However, in spite of the presence of more calcium than was theoretically sufficient to precipitate the phosphate, lower colorimeter readings were always obtained when the original medium contained high concentrations of phosphate. For these experiments, therefore, the standard solutions always contained phosphate concentrations equal to those in the unknowns. In view of the high buffering action of hydroxylamine, the addition by Lipmann and Tuttle of a small amount of acetate buffer to the reaction mixture was pointless and was omitted here. The method finally adopted was as follows.

Two ml. of tissue suspension was added to 2 ml. of $2M$ hydroxylamine, adjusted to pH 6.5 to 6.6, in a 15 ml. centrifuge tube and the mixture was allowed to stand for 10 min. with occasional stirring. Then 1 ml. of $0.6N$ sodium hydroxide was added to bring the pH to about 7.0, 2 ml. of 3.4% zinc chloride − 5.6% calcium chloride solution was added with stirring and finally 2 ml. more of $0.6N$ sodium hydroxide was added slowly with stirring. After centrifuging, 4 or 5 ml. of the clear supernatant fluid was treated with 1 ml. each of $3N$ hydrochloric acid and 5% ferric chloride in $0.1N$ hydrochloric acid. After standing for 5 to 30 min. the color was determined with the Klett-Summerson colorimeter with filter No. 540. Recoveries of succinhydroxamic acid standard solutions added to brain tissue suspensions in either Ringer-phosphate or $0.11M$ phosphate ranged from 94 to 106%.

Dr. Lipmann has since informed us that a considerably lower concentration of hydroxylamine may be used.

Results

In Table II results of acetic acid determinations before and after incubation of isotonic suspensions are shown. The amounts of acetate formed varied but the average in the presence of added pyruvate is in accord with the amount formed after two hours' incubation, 9.9 μmoles per gram, calculated from the results of Elliott *et al.* (8). Without added pyruvate, smaller

TABLE II

PRODUCTION OF ACETIC ACID IN ISOTONIC RAT BRAIN SUSPENSIONS

Micromoles of acetic acid per gram of whole brain; incubation one hour at 38° C.

Substrate	Initial	In-cubated	Increase	Substrate	Incubated	Increase* (average)
Pyruvate, 0.025 *M*	—	10.6	—	Pyruvate,	11.5, 9.2, 8.9	8.3
	0.6	7.5	6.9	0.25 *M* in		
	0.5	6.3	5.8	0.11 *M*		
	0.5	9.7	9.2	phosphate		
	3.9	8.7	4.8			
	1.8	5.4	3.6	Glucose, 0.01 *M.*	2.2, 6.3, 5.3, 5.0	3.2
	2.0	6.8	4.6			
	0.5, 0.7, 2.2, 3.2	—				
Average	1.6	7.8	6.2	No addition	6.2, 4.4, 3.8, 5.0	3.2
No addition (insulinized)	0.6	1.9	1.3			

* *After deducting average initial figure.*

amounts of acetate are formed. This is probably produced from the traces of pyruvate that are present in the respiring tissue as an intermediate in glucose or lactate oxidation. Fresh brain suspensions, even without added substrate, always contain appreciable amounts of lactate formed from residual blood glucose. The amount of lactate, and hence of acetate formed, is much reduced in brain from insulinized rats (8, 10).

Acyl Phosphate

Results of acyl phosphate determinations are shown in Table III. The amounts of acyl phosphate present were very small and often below the range of accuracy of the determination. The amount of acyl phosphate found did not increase with time of incubation but rather decreased and was considerably less than the equivalent of the acetic acid usually found. It is evident therefore that the substance estimated as acetic acid is not produced from acetyl phosphate by hydrolysis in the course of the acetic acid determination. Lipmann (13) has found a very active acyl phosphatase in many tissues that splits acetyl phosphate to acetic acid and phosphate. Experiments with the brain suspensions showed that about 70% of added lithium acetyl phosphate,*

* *Kindly given to us by Dr. Fritz Lipmann.*

TABLE III

ACYL PHOSPHATE IN RAT BRAIN SUSPENSIONS

Micromoles of acyl phosphate* per gram

Substrate	No. of expts., averaged	Incubation period		
		Initial	30 min.	60 min.
Pyruvate, 0.025 M	3	2.3	1.4	1.3
Glucose, 0.01 M	5	3.2	3.1	2.3
No addition	3	1.7	1.4	1.2
Pyruvate, 0.025 M in 0.11 M phosphate medium	3	1.5	1.1	1.1

* *Figures are only approximate since a number of results fell below 1.5, which is about the lower limit of reliability of the method.*

0.007 M, was split in five minutes at 38° C. It therefore seemed possible that acetyl phosphate might be formed by the tissue but be hydrolyzed during the incubation period. Lipmann (13) and Shapiro and Wertheimer (19) have shown that acyl phosphatase is strongly inhibited by high concentrations of phosphate. This was confirmed with brain suspensions in isotonic, 0.11 M, phosphate in which the rate of destruction of added acetyl phosphate was decreased by about 85%. Brain suspensions in isotonic phosphate with added pyruvate produced acetic acid at least as fast as in Ringer-phosphate medium (see Table II) but again no sign of increased acyl phosphate was found. It thus seems unlikely that acetyl phosphate is an intermediate in the production of acetate by brain tissue. Small amounts of a substance that is estimated as acyl phosphate, about 2.4 μmoles per gram (average of seven determinations), were found even when the tissue was homogenized directly in medium containing 2M hydroxylamine to trap acyl phosphate already present in the tissue. It is possible that the substance estimated in this case is phosphoglyceryl phosphate, which also forms a hydroxamic acid yielding color with ferric salt, and which might be present in traces in tissue in which glycolysis is proceeding or has recently occurred. The amounts are so small and erratic, however, that they may represent nonspecific interference with the method by tissue materials.

The oxygen uptake rate of isotonic brain suspensions even in the presence of 0.11M phosphate was not affected by the addition of lithium acetyl phosphate, 0.007M.

Effects of Various Additions on Acetate Formation

In Table IV the effects of various factors on acetate formation are shown. Malonate addition increased the acetate production from pyruvate definitely and considerably. Malonate had only a faint inhibitory effect, about 5%, on the oxygen uptake rate of isotonic suspensions in the presence of added pyruvate or glucose. Addition of small amounts of malate or α-keto-glutarate

TABLE IV

EFFECT OF VARIOUS FACTORS ON ACETATE PRODUCTION

Micromoles of acetic acid per gram; incubation one hour at 38° C.

Isotonic suspensions		Hypotonic suspensions	
Additions	Acetate formed*	Additions	Acetate formed*
Pyruvate, 0.025 M	6.2 (average**)	Pyruvate, 0.025 M	9.0, 6.5, 7.5, 14.2
Pyruvate and malonate, 0.02 M	12.1, 11.0, 16.7, 14.6, 13.4	Glucose, 0.01 M	3.1, 9.6, 5.0
Pyruvate and malate, 0.005 M	5.5, 10.7, 9.1, 11.8	Pyruvate and malonate, 0.02 M	13.2, 13.9
Pyruvate and α-keto-glutarate, 0.005 M	10.6, 12.3	Pyruvate and malate, 0.005 M	7.5, 8.1
Pyruvate and thiamine, 0.003 M	6.5, 11.9, 9.4, 10.1	Pyruvate and malate and malonate	9.5, 10.6

* *After deducting average initial figure.*
** *From Table II.*

also seemed to increase the acetate production of isotonic suspensions slightly. (In previous work on similar suspensions (7) it was found that malate and fumarate produced an initial small increase in the oxygen uptake rate.) The addition of thiamine also seemed to increase acetate formation. Omission of magnesium ion from the medium or a fivefold increase in magnesium concentration had no obvious effects. With tissue in which the respiratory system was disrupted, by homogenization in hypotonic medium (7), the average amount of acetate produced was slightly increased but statistically the increase was of doubtful significance.

The addition of 0.02 to 0.1M fluoroacetate* caused no appreciable inhibition of the oxygen uptake of rat brain suspensions in the presence of either glucose or pyruvate. (Bartlett and Barron (1) found slight inhibition of respiration of rabbit brain slices but considerable, 53%, inhibition with guinea pig brain slices, which, they state, can oxidize acetic acid.) The acetate determination showed the presence of large variable amounts of steam-volatile acid. However, it was found that fluoroacetic acid is appreciably volatile with steam and causes large and variable blank titrations when it is present in the solution distilled. Three pairs of acetate determinations on tissue suspensions incubated with pyruvate and 0.02M fluoroacetate gave total 'acetate' values varying from 11.3 to 29.5 μmoles, averaging 19.7 μmoles per gram. Three pairs of blank determinations in which the pyruvate and fluoroacetate were incubated in the absence of tissue gave values varying from 8.4 to 18.6, averaging 10.9 μmoles, in the amount of solution that would contain 1 gm. of tissue. The amount of acetate formed with tissue present might thus be about 8.8 μmoles, the difference between these averages.

* *We are grateful to Dr. E. S. G. Barron for the gift of a sample of sodium monofluoroacetate.*

This amount is not very different from that formed with pyruvate and tissue alone and we cannot conclude from these results that fluoroacetate has a significant effect on acetate formation in rat brain tissue suspensions. Similar results were obtained with glucose as substrate and with $0.1M$ fluoroacetate with glucose or pyruvate.

Nonenzymic Formation of Acetate

Pyruvic acid is a highly reactive substance. Among other transformations that it has been reported to undergo is a decomposition to acetic acid on warming to 50° C. in dilute sodium carbonate solution (9). It was found that even in the extremely mild conditions here used, one hour at pH about 7.4 and 38° C., a slight amount of volatile acid is produced in the absence of tissue (see Table V).

TABLE V

NONENZYMIC FORMATION OF ACETATE FROM PYRUVATE

Incubated for one hour at 38° C., pH 7.2 to 7.4

Medium	Pyruvate added, μmoles/ml.	Ascorbate added, μmoles/ml.	Acetate formed	
			μmoles/ml.	Equivalent μmoles per gm. tissue, average
Ringer-phosphate	25	—	0.70, 0.36, 0.23, 0.05	2.2
"	25	15	3.4, 4.1	25.1
Tissue suspension	25	15	3.6, 2.7	20.7
Redistilled water	25	15	2.8, 6.0	29.1
Ringer-phosphate and 0.02 M malonate	25	15	4.9	29.8
Ringer-phosphate	12	30	7.9	47.6
"	25	0.3	0.2, 0.4, 0.6	2.7
"	1	—	0.3, 0.18, 0.12	1.0
"	1	0.3	0.11, 0.12, 0.25, 0.63	1.9

Cavallini (5) reported that, in the presence of ascorbic acid, pyruvate is oxidized to acetate and carbon dioxide in the acid to neutral pH range. In the abstract of Cavallini's work available to us, there is no suggestion of the mechanism of this reaction. Ascorbate is readily oxidized especially in the presence of traces of copper ion. Hydrogen peroxide, which is formed in the oxidation of ascorbate, would oxidize pyruvate to acetate and carbon dioxide. Alternatively, dehydroascorbate might react with the pyruvate. Results shown in Table IV confirm the report of Cavallini. Relatively high concentrations of pyruvate, $0.025M$, and ascorbate, $0.015M$, incubated together in neutral aqueous or Ringer-phosphate solution, yielded considerable amounts of a substance estimated as acetate. This occurred in the presence or absence

of tissue and in experiments in which the reactants were dissolved in water that had been twice redistilled in glass apparatus to minimize contamination with copper. Rat brain contains about 0.36 mgm. ascorbic acid per gram (2). A suspension containing 150 mgm. brain tissue per ml. might therefore contain $0.0003M$ ascorbate. Such a low concentration was not found appreciably to affect the amount of acetate formed from pyruvate in the absence of tissue.

Discussion

It thus appears that spontaneous breakdown and perhaps, to a slight extent, the reaction with ascorbic acid, could account for a considerable fraction of the acetate production in tissue suspensions. When metabolic processes are disrupted by the addition of malonate or by homogenization in hypotonic medium the accumulation of acetate is usually increased. This suggests the possibility that formation of free acetic acid by brain tissue is not a normal physiological process but may result from abnormal conditions existing *in vitro*. The results with fluoroacetate, an inhibitor of acetate oxidation in various organisms, do not suggest that acetic acid is formed and then further oxidized to any considerable extent. This agrees with previous results showing that added acetate is not metabolized by rat brain (6).

The possibly significant increases in acetate formation in the presence of malate and α-keto-glutarate suggest that these give rise to an increased concentration of an intermediate from which acetate may be formed. The apparent effect of added thiamine suggests that it may be concerned in the production of this intermediate or in a process that can produce acetate. However, it is probable that work with thoroughly disintegrated tissue or with tissue extracts, in which the respiratory systems are disrupted and the effects of various additions are more marked (see for example, Reiner (18)) would be necessary to clarify the mechanism of acetate production.

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THE INGESTION OF SEA WATER AS A MEANS OF ATTENUATING FRESH WATER RATIONS[1]

By MORLEY G. WHILLANS[2] AND GEORGE F. M. SMITH[3]

Abstract

Though the drinking of undiluted sea water by the castaway has been shown to be dangerous, there have been few studies on the use of sea water diluted with fresh water. The experiment was designed to permit a statistical evaluation of relative gain or relative loss in body weights to human subjects, from drinking a daily supplement of sea water. The addition of 10 oz. of sea water to the daily ration of 16 oz. of fresh water resulted in a mean (corrected) weight saving of 2.24 lb. over a period of six days. This result was significant. That this relative gain in weight was beneficial is supported by the subjects' unanimous opinion to this effect, by the specific gravities of blood and urine, and by the figures for blood and urine electrolytes in control and experimental subjects.

Introduction

The purpose of this investigation was to determine the possible value of drinking sea water with fresh water rations, as a means of retarding dehydration. The experiment was so designed that a statistical evaluation of the changes in body weight in the treated and control groups was possible (Fisher (5)).

The drinking of undiluted sea water by the castaway has been shown, beyond reasonable doubt, to be a dangerous procedure. The records of survivors from sea disasters and data obtained in the laboratory are in agreement. Elkinton and Taffel from their studies on dogs (3) and Winkler and his co-workers from studies on humans (11) concluded that the intracellular dehydration accompanying the drinking of sea water was progressive, though the extracellular fluid tended to be maintained. A similar conclusion was reached by Gamble (8),—that though salt (sea) water will assist in the removal of solutes (including nitrogenous waste products) it does so at the expense of intracellular fluid. Elkinton and Winkler (4) in reviewing their work on the effects of drinking undiluted sea water, felt that no value could be expected from the use of diluted sea water.

Rectal administration of sea water had been suggested as a means of combating dehydration, the theory being that the colon is capable of selective absorption of water from its contents. It was shown (1, 7), however, that the colon did not concentrate sea water, and that this method was inadvisable as dehydration was furthered by the procedure.

A frequent objection advanced against drinking sea water is that the strongest solution of sodium chloride the kidneys can excrete is slightly less than 2% (2), whereas sea water contains approximately 3% of sodium chloride.

The arguments in favor of drinking sea water relate to the fact that men on short water rations are in chloride debt as the result of the continued loss of chloride in the urine and sweat. There is also a loss of sodium with the chloride, and the depletion of the extracellular fluid volume that results may be the important determining factor in survival time.

In an evaluation of the experience of five survivors who had been adrift for 15 days, Good (9) suggests that "adding small amounts of salt water to the daily ration of fresh water so that the potability of the water is not changed, seems to be an excellent idea as it will make the water last longer, will tend to keep the blood chlorides at a normal level, and should have a tendency to satiate thirst." The five survivors he studied had added a small amount (actual amount not specified) of sea water to their daily ration of fresh water for 13 days. For the last two days there was no fresh water. Two of the survivors then drank undiluted sea water and subsequently wanted to cut their own wrists.

Ladell's studies on humans (10) indicate that there is a slight but definite relative gain in body weight from drinking diluted sea water. He also pointed out that 400 cc. of sea water daily is the maximum amount that can be taken into the body, without calling on the body's reserves to excrete or to retain salt. However, the British Medical Research Council in taking account of factors not specified, advised against the drinking of sea water, although soaking biscuits in it was permitted. The United States Army Air Forces' manual *Survival* recommends the mixture of one part of sea water in six parts of fresh water, as a means only of replacing salt lost by sweating during hot weather.

A Russian naval officer who survived 36 days in a small boat in the Black Sea reports (12) that he and three other men with him drank sea water along with their small ration of fresh water and what they could obtain from rain. Though he was the only man who survived (the others died on the 19th, 24th, and 30th days) he states that they became accustomed to drinking the sea water, and that two years later he finds that fresh water and ordinary food still taste undersalted.

Material

Human volunteer subjects were used in all experiments herein reported. A number of preliminary studies were performed that showed that no more than an equal volume of sea water with fresh water could be tolerated for more than one day, and that even this concentration was apt to cause nausea. As a result of these studies a proportion of 10 oz. of sea water to 16 oz. of fresh water per day was set for subsequent experiments. The two controlled experiments were conducted with six airmen (three in each experiment) together with one medical or medical associate officer acting both as subject

and 'guide' during each experiment, giving a total of 48 test days. Another earlier test, involving four men for six days, was performed, but owing to a difference in the arrangement of the test procedure, it was not included in the final analysis. All the evidence secured from the results of this test pointed to conclusions similar to those obtained from the two tests herewith reported. The selection of subjects was made after a thorough physical examination and history-taking. Those passing the examination were then selected by alphabetical priority.

All experiments were performed in a 'tropical room.' Toilet facilities were provided in the chamber. During the day, from 9 a.m. to 6 p.m. the temperature was kept at 30° C., with water vapor pressure at 20 mm. Hg. At night the dry bulb temperature was 20° C. and water vapor pressure was 13 mm. Hg – typical of a 'tropical oceanic' environment. Air movement was supplied through multiple apertures in one wall, and a rate of 5 m.p.h. was maintained daily for four to six hours. The air was conditioned outside the room, and a complete change of air was effected approximately every five minutes. The careful control of temperature and humidity provided a constant, standard environmental stress, the same for each subject and the same for each experimental period.

The fresh water provided was the emergency canned ration, standard in emergency kits in the R.C.A.F. and R.C.N. One can of fresh water daily was allotted to each man, and though 16 oz. was the amount each can was supposed to contain, actually by measurement each contained 450 cc. ± 2 cc, The sea water was obtained off the coast of New Brunswick and on analysis showed a chlorinity of 18.0 gm. per milliter (2.95% sodium chloride, or 507.0 m.e. per liter of chloride ion).

The food consumed was the R.C.A.F. dinghy ration Type C, comprising four 1-oz. chocolate bars, two 2-oz. packages of biscuit, lemon-flavored boiled candies, sugar cubes, and two packages of chewing gum. The caloric content of this ration is 1088 cal. and because three-quarters of the ration was consumed per day, the daily caloric intake of each subject was 816 cal. The sodium chloride content of the ration was found to be 1.88 gm. per package, so that the daily intake of sodium chloride from food was approximately 1.4 gm.

Clothing consisted of shorts, cotton shirt, and low canvas shoes or slippers for all subjects.

Methods

The experimental design was arranged to show differences in weight loss between subjects who received only the basic food and fresh water ration, and those who, in addition, received sea water. Any significant difference in the loss of weight of the two groups could then be considered to be due to differences in water storage or loss. Each subject remained in the chamber for seven days and nights continuously.

Four subjects at a time were studied and a total of eight subjects were employed, there being two experiments. *Two subjects in each experiment*

received 16 oz. of fresh water per day (plus food rations) and the other two received 16 oz. of fresh water and 10 oz. of sea water per day (plus food rations). Selection into 'sea water' and 'fresh water' (or 'control') groups was made, by tossing a coin, after entry into the chamber. Nude weights of all subjects were taken on hospital balance scales and were recorded to the nearest $\frac{1}{8}$ lb. (2 oz.) every two hours during waking hours. All urine voided was measured volumetrically, tested for reaction, blood (first morning sample only), albumin, ketone bodies, and specific gravity.

The subjects were allowed to take their water rations in the manner they found most suitable to their taste, the only provision being that the water was to be taken in divided doses during the day. The most commonly used method was to drink the sea water while eating the rations, and to finish off the meal with a drink of fresh water.

On the morning the subjects entered the chamber they had had neither food nor water for at least nine hours. Nothing was taken by mouth for another 24 hr. so that when the test period began, on the morning of the following day, all subjects were in water debt to an approximately equal degree.

During the first experiment the freezing points of the first morning samples of urine were determined, for estimation of total solutes. Each urine sample was retained in a labelled bottle until all determinations had been completed. The chloride ion concentration of each sample was determined. For the sodium and potassium estimations aliquot samples of each urine specimen of each day were mixed, to make representative samples for each day for each subject.

Blood samples were obtained before 'breakfast' each morning from each subject, and specific gravity, hematocrit, chloride, nonprotein nitrogen, and sodium estimations made on each.* Blood potassium studies were also planned, but could not be completed.

Close attention was given to the prevention of boredom in the subjects. Cards, a radio, a generous supply of books and magazines, and almost daily motion picture showings in the chamber were provided. Each man was carefully impressed with the necessity for consuming all and no more than his allowance of rations and water, and for reporting any deviations, however small. Each man kept a diary in which he recorded all relevant data respecting himself and his fellow subjects.

Results

The results of two experiments, each involving four men for a test period of six days (48 test days) were used as material.

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* *Blood and urine sodiums were estimated by the wet ash method; the sodium was precipitated with zinc uranylacetate reagent; the color developed on solution of the precipitate was read by means of an Evelyn photoelectric colorimeter.*

Urine potassiums. After ashing the sample, the potassium was precipitated with chloroplatinic acid. The precipitate was then washed free of excess platinic acid with absolute alcohol. The amount of iodine set free by the addition of potassium iodide was then read with the photoelectric colorimeter.

Body Weight

It will be seen from Table I that the relative gain in body weight to the sea water group by comparison with the fresh water group falls within the generally accepted limits of statistical significance (P = 0.05).

TABLE I

ANALYSIS OF VARIANCE AND COVARIANCE OF INITIAL WEIGHT (X) AND FINAL
WEIGHT (Y) FOR TEST PERIOD OF SIX DAYS

Control group (weight in pounds)			Sea water group (weight in pounds)		
Subject	Initial wt. (X)	Final wt. (Y)	Subject	Initial wt. (X)	Final wt. (Y)
M	175.25	165.25	D	129.25	123.00
McL	160.75	148.75	G	138.00	131.25
S	150.50	142.00	L	116.00	111.75
F	137.25	129.25	K	151.25	144.88

Sums of squares and products of deviation about the mean

	X^2	XY	Y^{2*}	D.f.*	Y^2 cor-rected	D.f.*	M.s.*	F*	P*
Within groups	1434.50	1339.78	1258.55	6	7.24	5	1.45	—	—
Between groups	947.14	825.35	687.25	1	10.74	1	10.74	7.42	0.05
Total	2431.64	2165.13	1945.80	7	17.98	6			

Mean corrected final weights

Control group	Sea water group	Difference	Significant difference for P = 0.05
135.89	138.13	2.24	2.19

     * *D.f. = degrees of freedom, M.s. = mean square, F = variance ratio, P = probability.*

     This table is a standard analysis of variance and covariance. The technique of, and theory behind this statistical method may be found in many standard statistical books such as Statistical Methods for Research Workers, Section 49.1 by R. A. Fisher (5), (Oliver & Boyd, 1938 and later). Essentially the method makes due correction for the lack of balance in the experiment caused by the unequal initial weights of the subjects. The corrected sum of squares for final weight corrected (Y² corrected) was computed from the dependence of final weight on initial weight in the "within groups" row, as the sums of squares and products in this row are independent of treatment. This correction involves the loss of one degree of freedom for estimating the significance of the difference of corrected final weights between groups, as this degree of freedom has been used to estimate the regression coefficient of final weight on initial weight. The probability (P) of the observed variance ratio (F) occurring by chance was obtained from Statistical Tables of Fisher and Yates (6).

Uneaten Rations

The total weight of rations not eaten by the control group was 15.0 oz. (subject McL., 13 oz.; M., 2 oz.; both in Expt. No. 1). (The sea water group ate all their rations.) If this weight is added to the final body weight

of the control group, the difference in the corrected weights for the control group would be about 2.0 lb. instead of 2.24 lb. (as calculated above), still close to the 5% limit of confidence. It can be argued that the whole weight of uneaten ration should not be added to the final body weights of the control subjects. An important fraction of the rations would be lost as carbon dioxide and water, and the protein-sparing action of the carbohydrates (resulting in less demand on water for removal of protein metabolites) would be offset largely or completely by the proteins and solutes present in the biscuits and chocolate.

Tables II and III incorporate the results of the various estimations performed on each subject, for each day of the test periods. Table II gives the data day by day for the first experiment, and Table III that for the second.

Water Balance

A study of the urine volumes of the two groups of subjects indicates that an appreciable quantity of the sea water ingested may have been retained in the body (Table IV).

If the sea water supplement had been excreted quantitatively the daily urine volume per man in the group receiving it might have been 702.9 cc. (295.7 cc. sea water + mean daily urine volume per man of control group), whereas it was actually 558.6 cc. (mean daily urine volume per man of sea water group), a presumptive gain of 145.3 cc. from each 295.7 cc. of sea water drunk. Evidence that this gain of water was beneficial is supported by the opinions expressed by the subjects themselves. (See below.)

Chloride Balance

There was no significant change in blood chloride levels in any subject during the tests, as determined from the blood samples taken each morning. There was a chloride debt in all controls (see Table V), the total intake of chloride ion for both test periods being 19.17 gm. and the total output 33.15 gm., a deficit of 13.98 gm. This deficit was further aggravated by loss of chloride through sweat, and in unacclimatized men in the environmental conditions set by the test, the estimated sweat chloride concentration would likely be at least 0.1%, accounting for at least several grams more. (No measurements of sweat chlorides were made.)

Though the loss of chloride through sweat operated to the disadvantage of the control group, it was advantageous to the sea water group by assisting the kidneys in ridding the body of the excess chloride ingested. As a group, the sea water subjects stored chloride during the test period, though during the final two days of the first experiment, Subjects D and G were actually excreting chloride in the urine faster than they were taking it in.

The rate of excretion of chloride ions by the control group of subjects fell to a rate of slightly more than 30 m.e. per day by the second day of the test period, where it remained for the remaining four days. The sea water group

TABLE II

LABORATORY DATA: FIRST EXPERIMENT

Subjects	Day of expt.	Urine							Whole blood				
		Daily vol., cc.	Sp. grav., 1st a.m. sample	Total solutes, 1st a.m. sample, mgm./100 cc.	Daily Cl ion, m.e.	Daily Na, m.e.	Daily K, m.e.	Cl ion, m.e. per liter	Na, m.e. per liter	Hematocrit	Hbg, %	Sp. grav.	NPN, mgm. %
Controls													
M	1	520	1025	5947	85.1	69.1	44.8	85.5	79.3	51.5	95.0	1.064	40
	2	410	1026	6351	43.0	29.3	40.6	84.9	79.3	52.0	97.0	1.064	38
	3	360	1025	5924	31.4	26.6	40.4	82.9	80.2	54.0	103.0	1.066	42
	4	380	1027	5884	29.3	30.1	44.7	96.1	77.6	52.5	101.0	1.067	45
	5	395	1028	6107	29.7	30.7	76.1	91.1	97.35	53.0	103.0	1.067	45
	6	355	1029	6403	33.4	34.6	47.9	84.2	82.0	51.5	101.7	1.066	47
	Total	2420			251.9	220.4	254.5						
McL	1	575	1023	6026	75.8	53.4	49.2	88.1	82.0	51.0	92.5	1.063	38
	2	345	1027	6053	39.1	27.8	39.3	99.3	82.0	51.0	95.0	1.062	37
	3	375	1029	6763	42.0	37.6	44.5	95.1	90.8	50.0	95.0	1.062	50
	4	360	1029	7337	43.0	40.3	42.3	87.5	82.0	50.0	93.8	1.063	49
	5	325	1027	6407	32.5	29.0	40.8	87.5	82.9	50.5	97.5	1.064	49
	6	500	1028	6683	34.6	45.1	52.1	82.8	81.1	51.2	97.0	1.064	45
	Total	2480			267.0	233.2	268.2						
'Sea water' subjects													
D	1	475	1021	5400	70.0	70.1	44.1	91.8	80.25	52.0	90.5	1.062	42
	2	720	1025	5652	161.5	106.5	65.4	86.9	82.9	49.5	90.5	1.062	33
	3	700	1022	5013	176.1	136.7	55.2	93.9	86.4	49.0	90.0	1.060	28
	4	565	1023	4798	152.0	127.4	44.1	87.6	76.7	49.0	91.3	1.061	37
	5	690	1025	5450	184.0	176.3	41.0	91.0	87.35	47.0	90.0	1.059	31
	6	720	1027	4966	184.1	168.0	46.2	87.6	82.0	51.0	97.0	1.062	—
	Total	3870			927.7	785.0	296.0						
G	1	514	1022	5388	70.9	42.1	58.0	87.0	79.3	52.5	97.5	1.063	45
	2	590	1022	5100	104.7	73.4	64.8	91.0		50.0	95.0	1.062	38
	3	630	1025	4698	164.5	136.7	59.9	96.0	81.7	51.0	98.3	1.064	38
	4	620	1023	5638	167.0	147.2	53.2	87.0	82.9	49.7	95.0	1.063	60
	5	630	1027	5042	175.8	158.3	47.1	87.5	82.0	51.5	99.0	1.063	45
	6	720	1030	5603	194.3	176.5	53.2	87.0	82.0	50.5	97.5	1.063	45
	Total	3704			877.2	734.2	336.2						

TABLE III

LABORATORY DATA: SECOND EXPERIMENT

Subjects	Day of expt.	Urine							Whole blood			
		Daily vol., cc.	Sp. grav., 1st a.m. sample	Daily Cl ion, m.e.	Daily Na, m.e.	Daily K, m.e.	Cl ion, m.e. per liter	Na, m.e. per liter	Hematocrit	Hbg, %	Sp. grav.	NPN, mgm. %
Controls F	1	580	1.023	55.9	31.3	58.0	87.0	72.2	56.5	99.8	1.065	40
	2	450	1.023	33.5	18.4	46.4	87.5	77.5	55.0	98.3	1.063	42
	3	385	1.023	21.8	24.6	33.7	87.5	75.8	56.0	100.2	1.064	40
	4	355	1.026	20.9	23.2	36.8	90.2	75.8	56.3	101.7	1.065	35
	5	330	1.027	35.9	16.7	37.1	84.9	71.5	57.0	102.5	1.065	42
	6	365	1.026	18.8	23.4	47.9	87.0	82.0	56.0	101.0	1.065	40
	Total	2465		186.8	137.6	259.9						
S	1	494	1.032	52.2	57.2	39.1	87.5	80.2	53.0	97.5	1.064	40
	2	427	1.026	49.5	28.3	30.2	87.5	82.9	51.0	95.8	1.063	40
	3	380	1.025	34.5	23.8	30.2	94.9	82.9	52.5	97.0	1.064	42
	4	387	1.024	33.6	31.0	30.9	89.6	78.4	53.0	100.2	1.066	35
	5	345	1.026	27.1	33.6	31.0	87.0	78.4	53.2	99.8	1.065	45
	6	375	1.029	28.8	31.9	36.6	86.3	85.5	51.5	97.5	1.064	42
	Total	2408		225.7	205.8	198.0						
'Sea water' subjects K	1	442	1.024	114.1	74.2	60.6	85.5	96.9	49.5	88.1	1.060	50
	2	378	1.025	85.1	62.4	27.1	85.0	85.5	48.5	88.1	1.060	31
	3	366	1.024	92.9	71.4	33.7	87.5	86.5	50.0	91.3	1.060	31
	4	465	1.024	130.5	117.1	35.8	89.6	86.5	48.5	90.0	1.060	35
	5	535	1.026	147.9	134.9	50.2	86.2	85.5	48.0	88.1	1.058	37
	6	482	1.024	133.0	121.3	38.8	89.0	91.0	47.5	88.1	1.059	38
	Total	2668		704.3	581.3	246.2						
L	1	600	1.019	104.1	75.3	51.4	87.5	82.0	50.0	91.3	1.061	40
	2	565	1.026	144.2	97.6	61.2	87.5	84.6	48.0	89.3	1.059	42
	3	495	1.028	130.5	106.6	45.9	90.5	85.5	49.0	88.8	1.059	37
	4	535	1.024	145.0	128.2	40.7	94.5	82.0	49.2	92.5	1.061	33
	5	510	1.024	150.1	114.0	41.8	93.1	81.1	50.5	93.8	1.061	42
	6	460	1.029	132.2	121.8	30.4	95.2	85.5	50.0	93.0	1.062	45
	Total	3165		806.1	643.5	271.4						

TABLE IV

URINE VOLUMES

	Daily urine volumes (cc.) : totals								Mean daily urine volume per subject during test period	S.d.
	'Dehydra-tion day'	1	2	3	4	5	6			
Control group	2810	2169	1632	1500	1482	1395	1595	407.2	± 71.0
'Sea water' group	2732	2031	2253	2191	2185	2365	2382	558.6	± 106.5

TABLE V

CHLORIDE BALANCE

Total m. e. of chloride ion for each group for all six days of test period

	Intake	Output (excluding loss in sweat and feces)
Control group	Rations 539.87	933.7
'Sea water' group	Rations 539.87 Sea water 3619.6	
	4159.47	3353.63

excreted an average of approximately 160 m. e. chloride ion per day during the last two days of the test period, slightly less than the amount ingested daily (Fig. 1). The maximum chloride ion concentration during the tests in any urine sample was 320.1 m.e. per liter representing approximately 1.9% of chloride salt.

There were no symptoms recorded by any subject or signs noted on physical examination that could with reasonable certainty be attributed to salt deficiency or salt storage.

Urine Sodium and Potassium

The sodium excretion in both groups of subjects closely paralleled the excretion of chlorides. The expected high urine sodium values in the group receiving sea water are clearly evident (Fig. 2). The excretion of sodium increased daily until the fifth day where it levelled off at a mean value of 146 m.e. The control group appeared to reach a steady rate of excretion around 30 m.e., closely tallying with the corresponding chloride excretion.

The excretion of *potassium* during the test period was greater by the group receiving sea water (17.2% more than the control group). However, the rate of excretion towards the end of the test period, as shown in the graph

(Fig. 3) was approximately the same in each, with the difference favoring the sea water group. From this, it appears that the sea water group lost more intracellular water during the first half of the test period, but in comparison with the control group they lost water from their cells at no greater rate during the final two days.
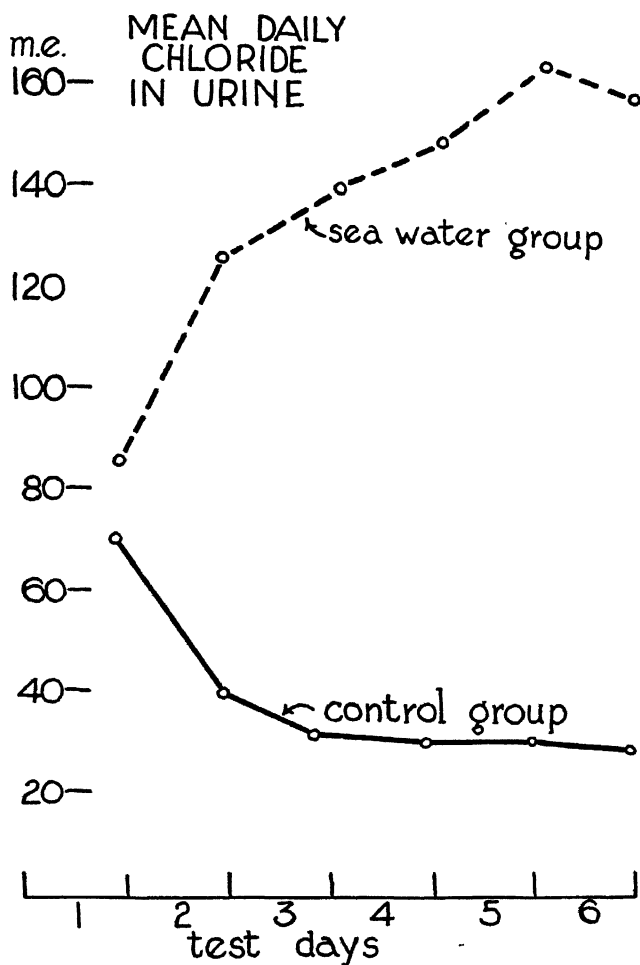
FIG. 1. *Mean daily chloride in urine. Each point represents the mean chlorine ion excretion of all four subjects in each group, for one day. The ordinate = m. e. of chloride ion in urine. The abscissa = test days.*

Blood Sodiums

The blood sodium levels remained within normal limits throughout both test periods, and there was no significant difference between the values for the control and sea water groups. (N.B.—The blood sodium and chloride values given in Tables II and III refer to *whole* blood determinations.)

Examination of Nitrogenous Metabolites

There was no evidence of retention of nitrogenous metabolites in either group of subjects, as revealed by the blood nonprotein nitrogen levels. As shown by the graphs, it is clear that in general, the subjects receiving sea water excreted the nitrogenous substances fully as satisfactorily as did the
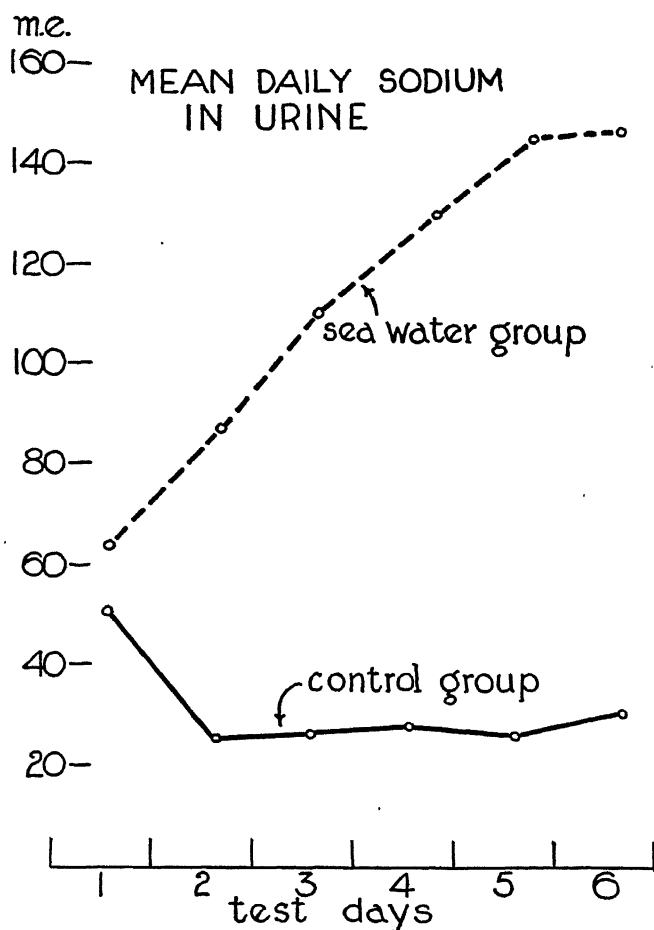
FIG. 2. *Mean daily sodium in urine. Each point represents the mean sodium ion excretion of all four subjects in each group, for one day. The ordinate = m. e. of sodium ion in urine. The abscissa = test days.*

control group. The highest reading (60 mgm. %*) was obtained on the morning of the fourth day of the first test period from a sea water subject (G). On that day he reported "0850: didn't sleep so well last night. My mouth is like the bottom of a bird cage. I am so—thirsty this morning." "1830 urine 160 cc. Felt—awful between 3 and 6 o'clock, but feel better now. Slightly weak and lack the pep that I had on Tuesday" (the day of dehydration preliminary to the test period). Examination at 5 p.m. showed nothing

Mgm. % = mgm. by weight of ion per 100 cc. of solvent.

unusual. The tongue was clean and the subject was alert and seemed in good spirits. All examinations, physical and laboratory, were noncontributory. The following and subsequent days the blood NPN estimation for this subject was 45 mgm. %.
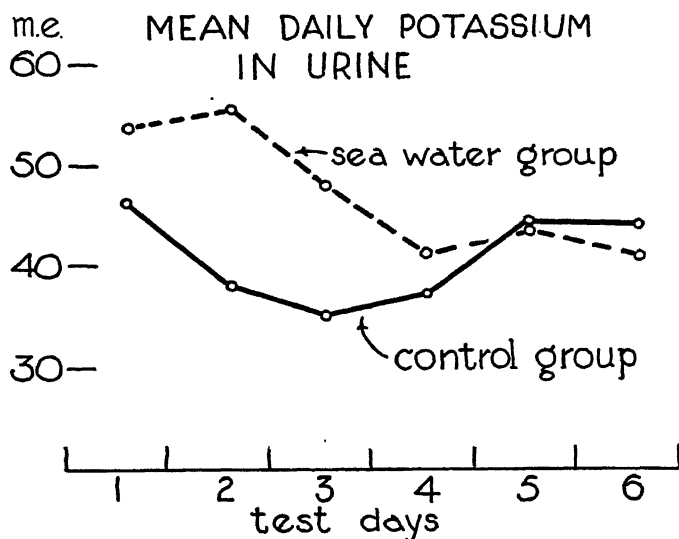
FIG. 3. *Mean daily potassium in urine. Each point represents the mean potassium ion excretion of all four subjects in each group, for one day. The ordinate = m. e. of potassium ion in urine. The abscissa = test days.*

Other Urine Examinations

In all subjects no evidence of renal damage appeared, as shown by tests for albumin and blood of all samples. The urine was generally acid in all subjects, though several men occasionally passed an alkaline sample. Ketone bodies were noted in three out of four subjects in the second experiment, reached a peak on the second day in the chamber (first day of the test period), and disappeared during the third day, never to reappear.

The urine specific gravity varied little between the two groups. During the first experiment, the average specific gravity of the urine of the sea water subjects was almost four points lower than that of the control group. The difference was in the same direction in the second experiment, but was small.

The highest specific gravity recorded was 1032 (fresh water subject S).

The percentage concentrations of total solutes in the urines of the fresh water subjects during the first experiment were approximately 1 gm. % higher than in the urines of those receiving the sea water supplement. However, the actual weight of solutes excreted by the sea water group was higher owing to the larger volume of urine put out.

Blood Specific Gravity, Hemoglobin, Hematocrit

The results of these three tests closely paralleled each other. The values, as shown in Tables II and III, rose appreciably during the test period and

fell to normal rapidly when the restrictions on diet and fluid intake were removed. The sea water supplement apparently was instrumental in preventing much rise in blood concentration and although, for instance, there was a rise of 3 to 5 in the hemoglobin percentages, the corresponding rise for the subjects on fresh water alone was 4 to 8 above the figures obtained before entry into the test room.

Consumption of Rations

At first there was little difficulty in consuming the food rations, but during the final two days of the experiment, the difficulty was extreme for all control subjects. Two of the control subjects were unable to eat their rations completely and for subject McL. this amounted to a total of 13 oz. for the last two days, and to 2.3 oz. for subject M. All items in the rations were rejected (chocolate and biscuits, as well as sugar and candy).*

The complete consumption of all sea water rations was an easy task by comparison with the difficulty of eating the food. One subject (K, on sea water) was a notable exception. He was not conscious of thirst during the test period. Opinion was divided as to whether sea water relieved thirst, but none said it made thirst more pronounced. Nausea was less prominent with the sea water subjects, but this, it is believed, was mainly because of the greater difficulty the control group had in eating their rations. Nausea was an invariable accompaniment of eating rations for the control subjects during the last two days of the test period.

Physical Condition of the Subjects

As no performance tests were included in the test program, almost all the evidence of the physical condition of the subject is necessarily qualitative in type.

There was no major change in the *appearance* of any of the subjects during the test period, and none appreciable until the second test day. By the fourth day, all subjects were showing evidences of weakness, and their faces were 'pinched' and pale. Posture and gait indicated loss of muscular tone and movements generally were rather slowly performed. These changes were thought to be less evident in the subjects receiving sea water. During the last two days of the test period, those who received sea water seemed more alert and less depressed.

The *saliva* became almost solid in consistency towards the end of the test, and had a distinctly foul taste and odor.

The blood pressure and pulse rates of subjects in the reclining and sitting postures were recorded at the same hour daily. Nothing significant of any abnormality was found.

Marked *constipation* was present in all subjects. Feces usually consisted of not more than three or four scybalous masses, 1 in. in diameter or less.

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* The great effort to consume the rations is exemplified by one subject. After choking down his 'meal', he repeatedly regurgitated it into his mouth. It was only by keeping his hand over his mouth that he prevented the loss of the rations.

Most of the subjects had no more than two bowel movements during the test period.

Subjective Data

Most complaints were of *thirst*.   Thirst was marked in degree in two control subjects, both of whom were so obsessed with it that they got out of bed during the fifth night and drank 2 oz. of their water rations for the following day.

Insomnia was troublesome for all subjects during the last three nights, and often appeared to be due to a gnawing pain in the abdomen (usually epigastric).

A sense of *weakness* was first noted by most subjects on the third test day. On the following day and subsequently, noticeable tiredness and lack of ambition was recorded by three of the control subjects and by one sea water subject.   In the first experiment the control subjects were convinced that they had appreciably less stamina than those who were drinking sea water. This view was expressed by one control subject as follows: "G and D (sea water subjects) both mentioned that they could last another week if they had to . . . . . .   I know, for one, that I would be a stretcher case if I had to stick it out another week and I think I can safely say that M (other control subject) feels the same way."   (M confirmed this in his diary.)

Boredom was a universal complaint during the last two test days, but never became an important problem.   There were no symptoms or signs of mental aberrance at any time during or following the test.

Recovery Period

The subjects reported for observation several times during the day of their release, the following morning, and one week later.   No untoward symptoms or signs appeared in any subject. · All showed a surprisingly rapid recovery. Within six hours after the first meal there was a noticeable return of normal skin color and alertness, together with a surge of buoyant cheerfulness.   No difference was detectable between the two groups of subjects in the length or character of their respective recovery periods.

Slight epigastric discomfort, consisting mainly of a feeling of distension and occasionally of burning was noted during the first day after leaving the chamber.   (This symptom probably was due to mechanical dilatation of the stomach by unaccustomed volumes of food and fluid.)

Value of Drinking Sea Water; Opinions Expressed by the Subjects

All subjects felt that the extra supplement of sea water resulted in less loss of stamina and that it facilitated the chewing and swallowing of the rations. All thought that if they were in a dinghy at sea and had only a small supply of fresh water, they would drink sea water with the fresh water rations.

Discussion

An experiment of this scope and character can only point the way to a more precise and more practical study of the problem.   The influence of seasickness, exposure to the weather, and mental stress may severally or in concert

render the use of sea water in any quantity inadvisable. From this experiment, it is evident that under the conditions of the test, some benefit without any important disadvantages was conferred by the addition of sea water to the fresh water rations. Further studies are desirable to improve the precision and predictability of the results, and it is clear that no instructions respecting the use of sea water should be formulated until actual trials in a dinghy at sea have been completed. In preparing for such trials, it would seem advisable to replace the sodium salts in the food rations with potassium salts, and thereby permit the castaway to make better use of the sea water supplement to his fresh water ration.

It appears to the authors that if men can be trusted to follow instructions to avoid sea water, they could also be trusted to follow instructions to utilize it to their best advantage.

Acknowledgments

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THE SPORES OF *HISTOPLASMA*[1]

BY ELEANOR SILVER DOWDING[2]

Abstract

Tuberculate spores distinguish the mold stage of *Histoplasma* from that of related pathogenic fungi. The tuberculations are not thickenings of the spore wall. They are extensions of the spore contents through the wall openings. The whole or the tip of a tuberculation may become detached and act as a spore. The yeastlike pathogenic phase of *Histoplasma* originates either from the hyphae, from the small spores (conidia), or from the tuberculations of the large spores (chlamydospores). *Histoplasma* can be recovered in culture from the lungs of white mice that have inhaled the spores. No chlamydospores have been discovered in sections of their lungs. Nevertheless, in districts of high histoplasmin sensitivity, when lungs with nodules are examined in routine autopsies, the finding of any spherical body with external spores might well suggest an arrested histoplasmosis.

Introduction

Formerly histoplasmosis was regarded as a regularly fatal disease. We suspect today that it also produces nonfatal respiratory conditions (14). Similarly, coccidioidomycosis was known earlier in its grave disseminated form and only later in its milder respiratory manifestations.

That *Coccidioidis immitis* is responsible for an influenzalike disease rests not alone upon indirect evidence, i.e., that many persons in the Southwest United States with lung calcifications are coccidioidin-sensitive (1). It also rests upon the following direct observations.

(1). Out of 3000 routine autopsies in San Francisco, Cox and Smith (5) found four lungs with hard nodules. The nodules contained spherules 15 to 40μ in diameter. One spherule, which had spores within it, was identical in size and structure with the fungus *Coccidioides immitis*. The spherules in the other lungs were probably also *C. immitis*. These bodies were found in persons who died of causes other than fungous infection.

(2). Many natives of California acquire San Joaquin fever and erythema nodosum. Dickson and Gifford (6) showed not only that these persons possess lung calcifications and that they are sensitive to the fungus extract coccidioidin, but also that the sputa and stomach washings of many of them contain spores of *C. immitis*.

In fact it is now proved that *C. immitis* produces a benign infection.

That *Histoplasma capsulatum* causes benign infection rests only upon indirect evidence, i.e., that many persons in East Central United States are

histoplasmin-sensitive (13). No spores of *Histoplasma* have yet been found in the lungs, sputum, or stomach washings of histoplasmin-sensitive persons.

It was thought, therefore, that a more intimate knowledge of the spores of *Histoplasma* in their saprophytic mold phase and in their transition to the parasitic yeast phase might be a step toward a better understanding of histoplasmosis.

Methods

For the microscopic study of spores, the usual method of transferring the mycelium by means of an inoculating loop to the mounting medium disarranges the hyphae and scatters the spores of *Histoplasma*. Therefore, the following method was employed.

A sterile cover slip was lowered over a Petri dish culture until it came into contact with the aerial mycelium. For immediate examination, the cover slip, with its adherent spores, was placed on a slide containing a drop of cotton blue and lactic acid (Fig. 1, *c*). For cultural study, it was sealed with vaseline upon a hollow-ground slide containing water, with or without the addition to the spore print of nutrient broth or agar (Fig. 1, *a* and *b*). The microcultures so made were left either at room temperature or at 37° C. for about a week. Sometimes individual spores were kept under observation under the microscope for several days and drawn with the camera lucida at intervals.

When a cover slip is brought into contact with a *Histoplasma* culture by the method described above, a deposit of spores and a mosaic of hyphae adhere to it. Most of the hyphae are dead and only faintly visible, but many of the conidia are still arranged on them in the original position (Fig. 1, *d*). Chlamydospores appear under transmitted light as dark globules because they are immersed in liquid drops (Fig. 1, *b*). On lowering the cover slip upon mounting medium, the drops on the chlamydospores are pressed out into smears containing conidia (Fig. 1, *c* and *d* and Fig. 3, *c*). When a microculture has remained for some days in water the mosaic of hyphal remnants disappears, leaving behind a network of mucilaginous-appearing strands (Fig. 2, *ad*).

In the network resulting from the disorganized mycelium were sometimes found rectangular, doubly-refringent bodies with re-entrant angles (Fig. 2, *ad*). These were similar in every respect to the 'mosaic fungus' frequently seen in epidermal scales from ringworm lesions. They are additional evidence that such mosaic structures are indeed disintegration products of fungous hyphae, as has been previously suggested from this laboratory (8).

When water or broth was added to the spore deposit and the microculture so made was kept under observation on the stage of the microscope, it was found that conidia and small hyphal fragments sometimes moved towards the chlamydospores. This agglutination phenomenon, although apparently of no biological significance, is a hindrance in the laboratory because it tends to obscure the origin of the conidia. Thus the appearance of a conidium close to the chlamydospores such as the one in Fig. 3, *e* (an arrow points to it)

leaves some doubt as to whether it arose from the tuberculation or from a neighboring hypha. Furthermore, by the method just described few of the spores ever became truly wet. In fact many of them remained in an air
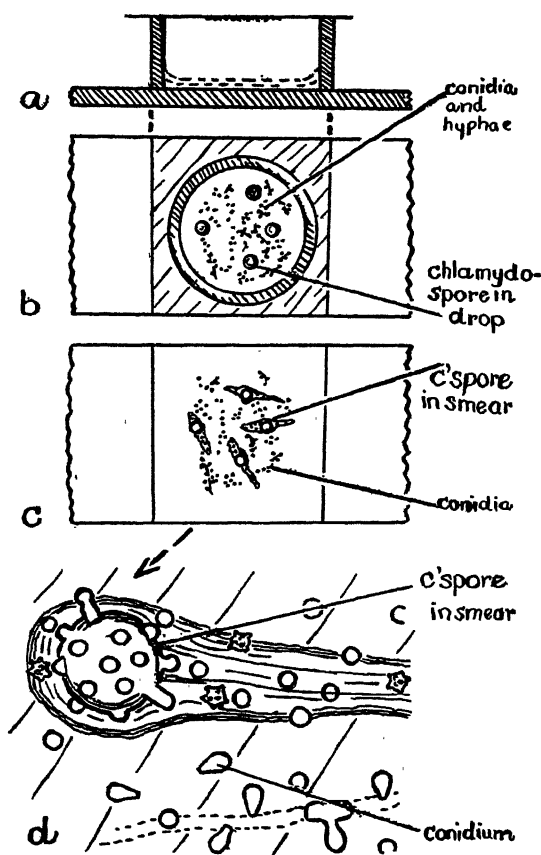
FIG. 1. *Diagrams of spore-deposit preparations. a, Van Tieghem cell, side view. b, The same from above, showing spores and hyphae. The chlamydospores are in liquid drops. c, The cover slip shown in a placed upon a slide bearing a drop of mounting fluid. The drops shown in b are spread out into streaks. d, Part of preparation c at higher magnification.*

bubble in the nutrient medium. For the study of the yeastlike phase particularly, it was necessary to insure (1) that the spores could not agglutinate, (2) that they became wet. The following method was used to bring this about.

By means of a pipette, the depression in a hollow-ground slide was almost filled with Sabouraud's medium. When the agar had solidified, a drop of plain broth was placed on top of it. The depression was then rimmed with vaseline. The cover slip, bearing its imprint of spores, was lowered, spores down, over the medium. The preparation was cautiously warmed, only enough to melt the vaseline. The cover slip then settled down on the moist
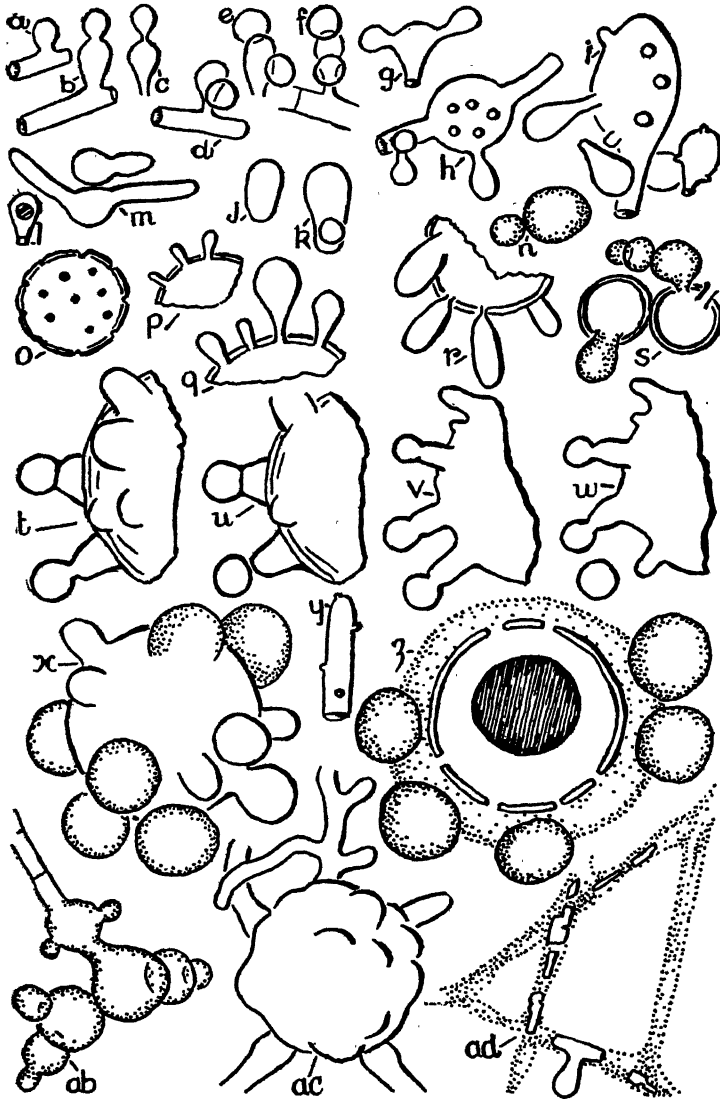
FIG. 2.   *Spores (all of H. capsulatum but r and s).   a to m, Conidia: b to k, secondary conidia; j, conidium; k, same drawn one hour later; l and m, stages in germination.   n, Yeast cell with one bud.   o to q, Development of a chlamydospore.   r, H. parvum, atypical chlamydospore.   s, B. dermatitidis, budding chlamydospore.   t, Part of a chlamydospore in moist air.   It is partly covered by mucilage but the tuberculations are exposed.   An upper one is septate, a lower one, constricted.   u, The same spore 13 hr. later.   The lower tuberculation has cut off a conidium.   v and w, Similar stages in another chlamydospore.   x, Incubated chlamydospore with tuberculations some of which have been converted into yeast cells.   y, Hypha with minute wall thickenings; z, Later stage of x showing yeast cells in a mucilaginous envelope surrounding chlamydospore. The darkly shaded body is an oil globule within the spore.   ab, The yeast cells developing from hypha.   ac, Germinating chlamydospore.   ad, Disorganized aerial hyphae with one conidium and mosaic of disintegration products.   Magnification: 1200.*

agar in such a way that no air bubbles broke the contact between the cover slip and medium. The film of broth became absorbed by the agar into which the spores settled. The preparation was put on a piece of bent glass tubing that was placed on a damp filter paper lying in the bottom of a Petri dish. The dish was kept for a week in an incubator set at 37° C.

The Spores

The spores of the mold phase of *Histoplasma capsulatum* have been described by Conant (2) essentially as follows: conidia are spherical to pyriform, smooth-walled, 2.5μ in diameter, either sessile upon the hyphae or borne on short lateral branches. The chlamydospores are at first smooth-walled but later show tuberculate sculpturings, and vary from the size of the conidia up to 15μ, the largest being covered with fingerlike protuberances sometimes 6μ long. The conidia and chlamydospores contain fat droplets.

It was found that not all strains of *Histoplasma* examined in this Laboratory produced spores. However, one strain, satisfactory in this respect, was obtained through the kindness of Dr. C. W. Emmons from the United States Public Health Service, and was used in the present investigation. The strain was similar to that described by Conant. Sometimes the spores and hyphae were not smooth but slightly rough (Fig. 2, *y*; Fig. 3, *a*).

When the spores of *Histoplasma* are in air their walls appear irregular and their contents are not visible, but in liquid the walls appear regular and the oil globules within show clearly (Fig. 2, *l* and *z*). The spores are difficult to wet. The chlamydospores, particularly, usually remain dry even when mounted in water or broth, a behavior due probably to the irregularity of their shape and to the secretion that envelops them. In Fig. 3, *a*, most of the spores are dry, in Fig. 4, *f* they are wet, and the matrix in which they are embedded is drawn out into mucilaginous strings.

Conidia

The conidia of *Histoplasma* are borne laterally upon the hyphae. Most of these hyphae are devoid of contents and their walls are only faintly visible (Fig. 3, *a*). On living hyphae, no scars or collarettes have ever been seen that might suggest that spores have been detached from them. It would seem that, usually at least, the conidia are set free by the disintegration of the aerial mycelium.

A conidium is capable of producing one or several secondary conidia as is shown in Fig. 2, *b* to *i*. A single conidium was kept under observation for two days and it was observed to produce a secondary spore during an interval of one hour (Fig. 2, *j* and *k*).

The distinction between conidium and chlamydospore is not clear-cut. Spherical hyphal swellings intermediate in size between them are often encountered. These also are capable of producing one to several secondary conidia (Fig. 2, *h* and *i*).

When a conidium is planted in broth and left for four days at room temperature it enlarges and grows out into one or two germ tubes (Fig. 2, *m*), which develop into a mycelium and repeat the saprophytic mold phase.

The mold stage may be converted to the yeast stage by the method previously described. A conidium planted in agar and left for three days at 37° C., enlarges considerably and forms a thin-walled yeast cell with granular contents (Fig. 2, *n*). Fig. 4, *b* shows conidia (with oil drops) and yeast cells. The yeast cell then either produces a short germ tube (pseudomycelium) or one to several buds (Fig. 4, *a*). These buds are widely variable in size, some of them being as small as the cells found in infected animal tissue and others measuring 7 μ (Fig. 4, *a* and *f*).

Chlamydospores

In two-weeks-old plate cultures of *Histoplasma*, chlamydospores are to be found among the younger peripheral hyphae. They may be intercalary or terminal. At this age they are smooth and thin-walled. Later, thickening is deposited unevenly upon the walls in such a way as to leave circular pits (Fig. 2, *o*). From the pits, fingerlike projections (tuberculations) extend.

The tuberculations are usually cylindrical, 2 to 3 μ in diameter, and sometimes constricted near the tips (Fig. 3, *b* and *d*). The terminal knobs are white, while the rest of the spore wall is brown (Fig. 3, *b* and *d*).

In cotton blue – lactic acid solution, the spore contents stain blue and plasmolyze slightly. In this medium, spore contents can be seen to extend in narrow strands from the spore itself through the wall openings into the tuberculations. *Therefore we may conclude that the tuberculations are not wall thickenings but that they are hollow and that their contents are continuous with that of the spore itself.*

Chlamydospores that were not yet tuberculate were planted in broth and left for a week at room temperature. They germinated (Fig. 2, *ac*) and developed into a new mycelium, repeating the saprophytic phase.

Tuberculate chlamydospores were left at room temperature in moist air and watched at successive intervals under the microscope. During an interval of 13 hr. it was found that tips of the tuberculations of two of them had become free from the spore (Fig. 2, *t* to *w*). It therefore appears that chlamydospores, like conidia, may produce secondary spores.

Tuberculate chlamydospores were left at 37° C. in agar using the method described earlier in the paper. After a day or two it was found that the tuberculations were no longer cylindrical but were pear-shaped or spherical,

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FIG. 3. *Spores developed in moist air. a, Deposit composed of mycelium most of it dead and only faintly visible; conidia, dry ones showing rough wall, wet ones showing oil globule within, one at lower right producing secondary conidia; chlamydospores, one to left with cylindrical tuberculations, one to right, later stage, with two conidia. b, c, d, and e, Chlamydospores that have been mounted in cotton blue. c, Mucilaginous smear with embedded spores probably originated from the chlamydospore. d, The tuberculations at the right with two young conidia arising as colorless spherical knobs, to the left faintly visible, a larger one. e, Older stages of d, at upper right a mature secondary conidium. Magnification: 1500.*

PLATE I

PLATE II
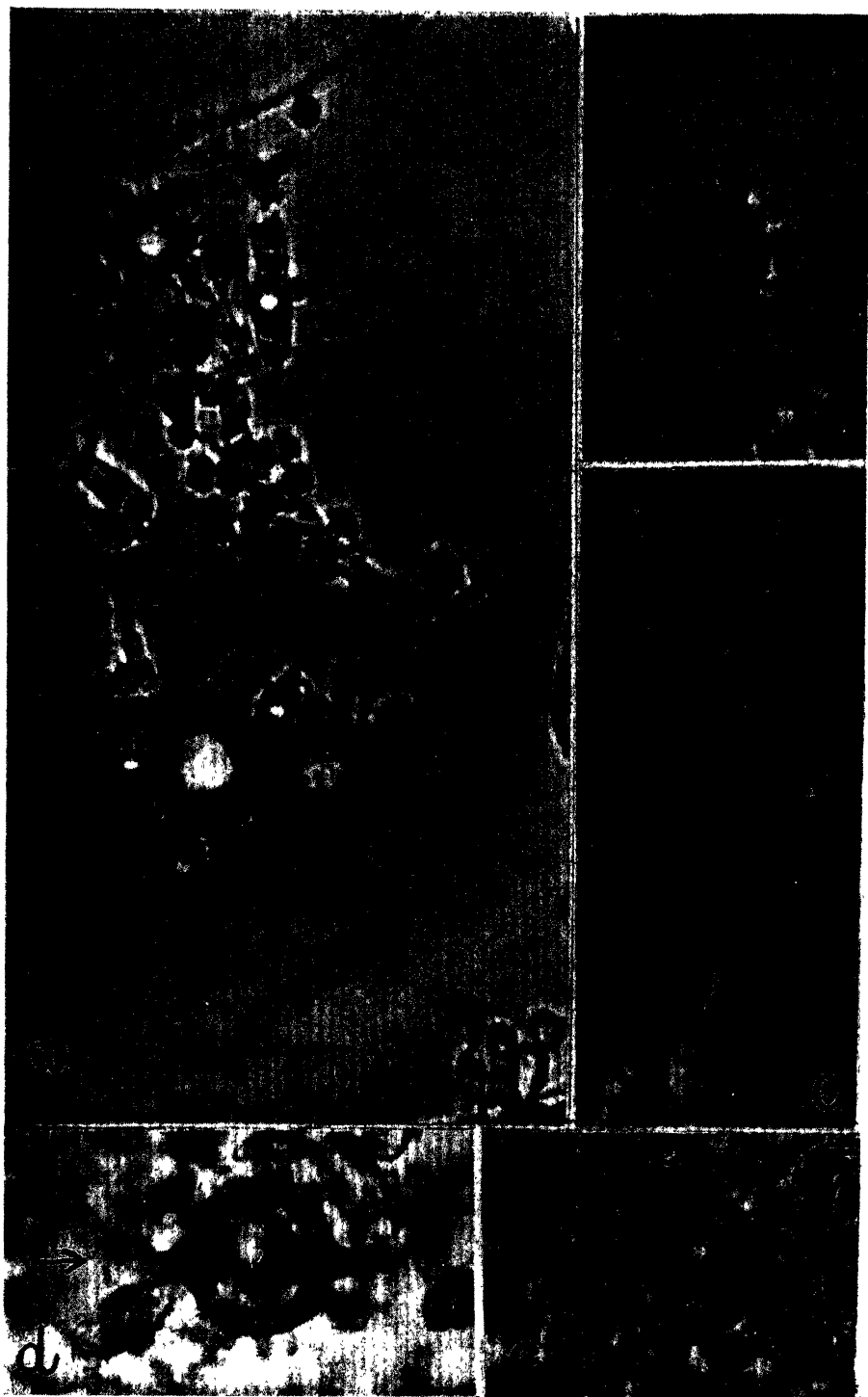
of the same size and shape as conidia and, like them, each contained a central globule (Fig. 4, *d*). We may safely say that they *are* secondary conidia.

After three days in the incubator, some of the secondary conidia begin to enlarge considerably. They are now thin-walled oval or spherical yeast cells (Fig. 2, *x* and Fig. 4, *c*, *d*, and *e*), indistinguishable from those arising from hyphae or conidia.

Fig. 2, *z* shows the final stage of the incubated chlamydospore. It is now devoid of tuberculations. Openings in the spore wall are all that remains to mark their place. The spore is empty except for a single oil globule and is surrounded by a mucilaginous sheath containing yeast cells. The reader's attention is directed to the remarkable similarity between this spore and a spore of *Blastomyces brasiliensis* Conant and Howell. Such a spore within infected tissue is shown in Fonseca and Almeida's photograph reproduced by Moore (12) (Fig. 6, *f*), and can be compared with the photograph of *H. capsulatum* to the left of it.

Three Genera Compared

Sometimes young cultures of *Histoplasma capsulatum, Haplosporangium parvum*, and *Blastomyces dermatitidis* possess a white tufted or prickly center and a mucoid periphery, and are all strongly similar. Sometimes strains of the three genera possess few if any spores to distinguish them. Their conidia, when they are produced, are not particularly diagnostic (Fig. 5, *b*, *d*, and *f*). Their chlamydospores are more distinctive, as can be seen from Fig. 5, *a*, *c*, and *e*.

The spores of *Haplosporangium* and of *Blastomyces*, which have been obtained in the laboratory by incubation, are known in animal tissue. The budding chlamydospores of *Histoplasma*, which the writer obtained by incubation, have not been described in animals. The only cells of *Histoplasma* known to pathologists are small intracellular oval ones 1 to 5μ in diameter (3). It is suggested that, in districts of high histoplasmin sensitivity, when lungs with nodules are examined in routine autopsies, the finding of any spherical body with external spores might be interpreted as a budding chlamydospore and might suggest an arrested histoplasmosis.

Histoplasma and Haplosporangium

Histoplasma capsulatum and Haplosporangium parvum, the mouse lung fungus, are similar in the following respects. (1). Both grow and produce spores when inoculated upon soil. (2). Their spores cannot be jarred loose easily

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FIG. 4. *The spores developed in agar at 37° C. a, Cells producing buds and at the upper left, pseudomycelium. b, To the right of the chlamydospore, conidia containing single oil drops, to the lower left a hypha bearing two conidia that have been converted into yeast cells, one of them budding. c, Chlamydospore producing conidia, those at the upper left enlarged to yeast cells. d, As c (the conidia being on the upper left surface of the chlamydospore, the yeast cells on its lower right). At the top of the picture is a yeast cell with two buds. e, Chlamydospore similar to c. f, Conidia, some on chlamydospore and some on disintegrated hyphae. At the bottom a yeast cell with five buds. Magnification: 1500.*

from the mycelium; they are not air-borne. (3). The spores of both are adhesive and can be transferred by contact. (4). In nature both fungi infect wild rodents (7, 9, 10). Further similarity is shown in the experiments now to be described.

A suspension of *Histoplasma* spores in sterile water was dropped on to the nostrils of six white mice so that they inhaled the drops. After the nasal inoculations, the animals were killed at intervals, ranging from one day to three weeks. From the lungs of three of the mice, cultures were obtained, which, from the appearance of their conidia (Fig. 6, *b*), and also their tuberculate chlamydospores, were identified with certainty as *H. capsulatum*. When the lungs of the six animals were sectioned and stained, however, no fungous spores were discovered in them.

A similar experiment was carried out with *Haplosporangium parvum*, this time using six wild white-footed deer mice trapped near Red Deer, Alta. It had been previously determined that in a control group of 33 animals from the same area only one animal was found with naturally occurring *Haplosporangium* infection. The deer mice were killed at intervals between one to five months after nasal inoculation. From the lungs of three of the six animals were obtained cultures that were identified with certainty as *H. parvum*. Furthermore, sections of the lungs of the same three animals showed the large chlamydospores characteristic of *H. parvum*.

The above experiments go to show that rodents may inhale the spores of *Histoplasma* and of *Haplosporangium* into their lungs.

Haplosporangium parvum was first discovered in 1942 in the squirrels, pocket mice, and kangaroo rats of Arizona (9). Subsequently it was found in deer mice in Alberta (7). Later, an organism that was similar to, if not identical with, *H. parvum* was described from a rock rabbit in Montana (11). Dr. Johnstone of the Department of Parasitology of the University of California School of Medicine showed the author similar bodies in his sections of lungs of a California water rat. Recent personal communications from other observers suggest that the fungus may also infect beaver in Minnesota* and muskrats in British Columbia.** It is evidently widely distributed over the western plains of North America.

Histoplasma capsulatum, isolated first from man, was in 1947 discovered in rodents. Its distribution is centered about the east-central United States, particularly Virginia (10). In Fig. 6, *c* and *d* are photographs of a bone-marrow smear from a sternal puncture of an Alberta Indian woman who

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** *Dr. I. McT. Cowan, Department of Zoology, University of British Columbia, Vancouver, B.C.*

FIG. 5. *Three genera compared. To the left, chlamydospores, to the right, conidia. a and b, Histoplasma capsulatum. c and d, Haplosporangium parvum. e and f, Blastomyces dermatitidis. Magnification: 1500.*

PLATE III

PLATE IV
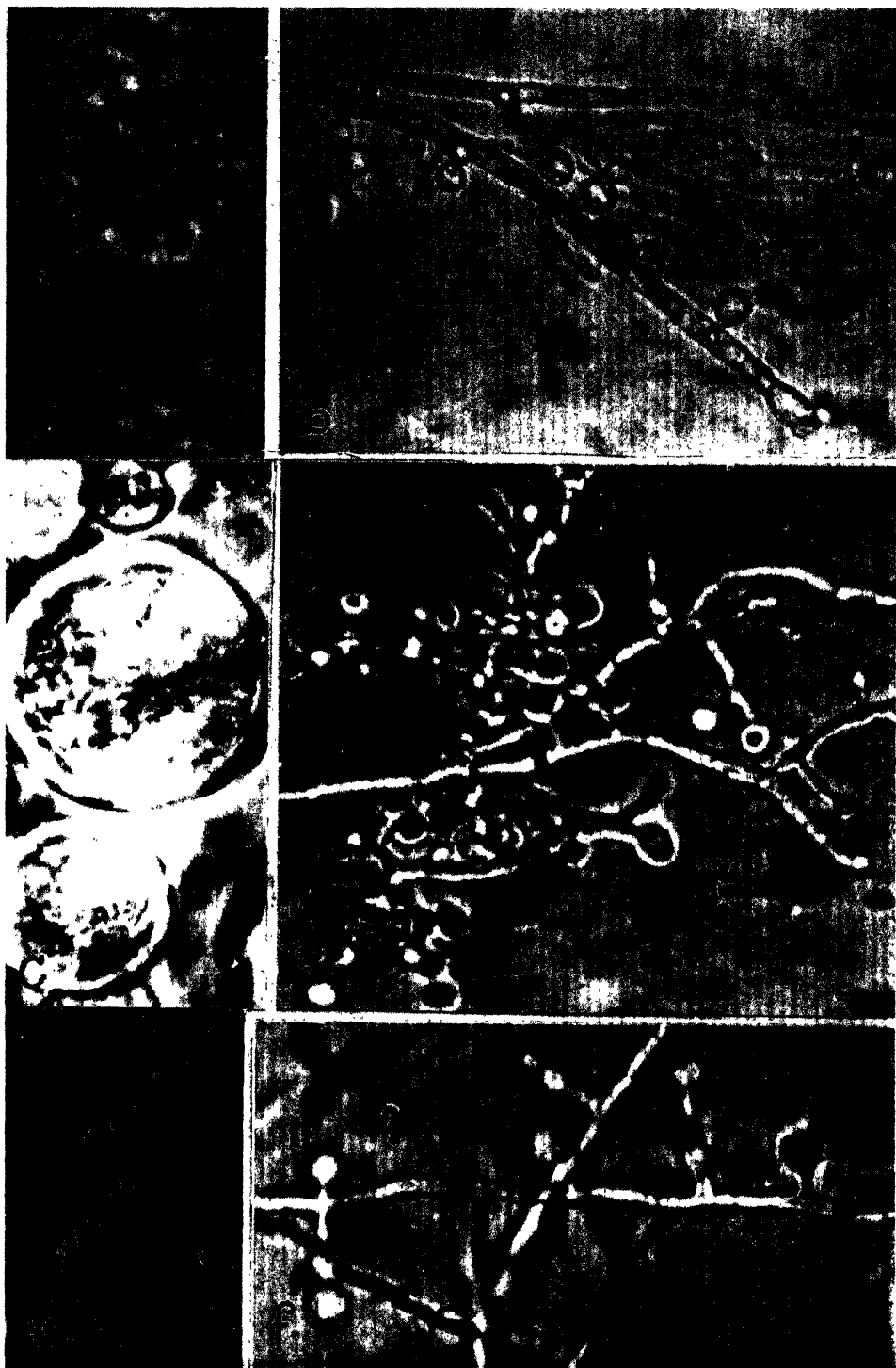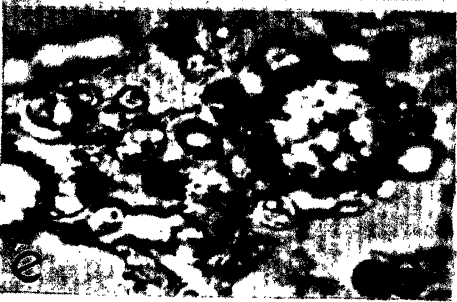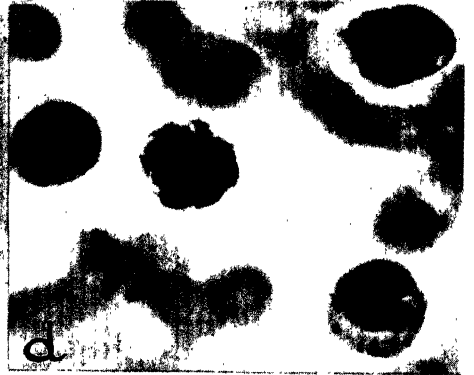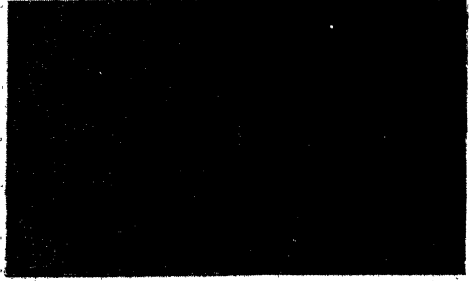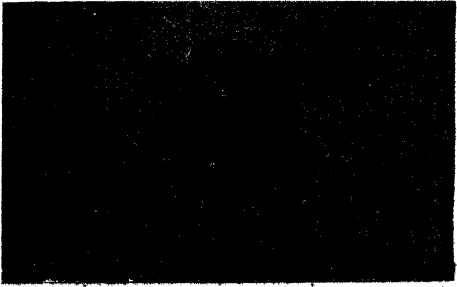
subsequently died, but not from fungous infection. The smear was sent to Dr. N. F. Conant of Duke University who agreed with the author that it contained spores of *H. capsulatum*. If our conclusion is correct, *H. capsulatum* is also more widely distributed than may have previously been supposed.

Histoplasma and Blastomyces

It has previously been shown (7) that the spores of *H. capsulatum* resemble those of *B. dermatitidis* in their appearance and behavior. Fig. 5, *a*, *b*, *e* and *f*, shows their conidia and chlamydospores. The chlamydospores of *B. dermatitidis* differ from the tuberculate ones of *H. capsulatum*. Nevertheless as can be seen from an examination of the lower spore in Fig. 5, *e*, they are sometimes unevenly thickened, and may grow out into extensions from the thinner parts of the wall, recalling those of *Histoplasma*.

More remarkable is the resemblance between *H. capsulatum* and *B. brasiliensis*. The yeast cells of *B. brasiliensis* reproduce by multiple budding (4) (Fig. 6, *f* and *h*). The chlamydospores of *H. capsulatum*, in transition to yeast cells, also produce multiple buds. The multiple budding forms of *H. capsulatum* and *B. brasiliensis* are so similar that the two fungi cannot be distinguished on the basis of these spores.

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FIG. 6.   *Histoplasma and Blastomyces.   a to e, and also g, H. capsulatum; f and n, B. brasiliensis: a, chlamydospore with conidia; b, mycelium and conidia recovered from mouse that had inhaled spores; c and d, smear from sternal puncture of native Albertan (photographs by Dr. N. E. Conant); e, budding chlamydospore; f, budding chlamydospore from tissue of South American patient (after Fonseca and Almeida); g, as e; h, budding chlamydospore in liver from case of· South American blastomycosis (after Conant).*

FAT ABSORPTION AND LUNG OIL[1]

By C. B. WELD[2].

Abstract

The lungs of normal animals and of normal animals fed with oil have been examined for the presence of oil. Frozen sections stained with Sudan IV and haematoxylin reveal Sudan stained intracellular granules and interstitial or intravascular oil globules in alveolar walls. In general these oil globules are larger and more numerous in the oil fed animals than in the normal controls. In the lungs of a considerable number of the animals absorbing fat, patchy areas of oil are found that resemble pulmonary fat embolism. Evidence is presented to show that the finding is neither a histological artefact, nor is it due to oil aspiration. Lung fat determinations give values higher in lungs showing marked oil deposits, but as a rule they remain within the normal range.

In the course of a study of the action of heparin on alimentary lipemia (8, 9) and of the possible use of heparin in pulmonary fat embolism (10), the observation was made that, in cats, relatively large oil globules, suggestive of pulmonary fat embolism, were present in the lung during alimentary lipemia.

In an attempt to obtain pulmonary fat embolism by *Clostridium Welchii* alpha toxin (10 and 2), a number of animals were injected with the toxin and lung specimens were taken. Occasional oil globules were found, possibly more than normal, but there was never a suggestion of clear-cut oil embolism. In the course of these experiments, the lung of a cat exhibiting alimentary lipemia was examined and was found to be loaded with oil. The globules were present interstitially, possibly in the capillaries, in the alveolar walls, and some in the alveolar spaces; they were patchily distributed, masses being present in some areas and none in other areas. The finding was so unexpected and seemed to have such little physiological reason that it was discounted. However, when the same finding (Figs. 1, 2, 3) was obtained in other animals it seemed necessary to accept it as a fact. The difference in appearance between the lungs from normal animals and the lipemic animals was striking. In the normal preparation, only an occasional small interstitial oil globule was seen. Further work was planned to confirm and extend the finding and to rule out the possibility of artefact.

Alimentary Lipemia

The animals were given oil by mouth. Cod liver oil or olive oil was trickled by syringe into the side of the mouth and was readily swallowed. An occasional animal exhibited some choking during the feeding; such animals were discarded. In general, dogs were given 20 cc. of oil or more, cats about 10 cc.,

and guinea pigs about 5 cc. Larger doses of oil sometimes give clearer results but sometimes seem to delay digestion and prevent the production of lipemia. Specimens of lung and blood were taken usually two to seven hours later. When possible, blood was taken from time to time to follow the course of the lipemia before lung specimens were taken. Some animals were given sodium pentobarbital after being fed the oil, and in some, early lung biopsy was done in order to obtain successive lung specimens from the same animal. Occasionally good lipemia was obtained in animals injected with pentobarbital but never was it obtained after biopsy. One of the disheartening features of these experiments is the inconstancy of the lipemia, which varies greatly in time of appearance and in degree, often not appearing at all. It was planned to obtain specimens of lung systematically at various stages of lipemia and it was hoped that a lung biopsy before lipemia would give a standard of comparison but it has not been possible. However there are examples of a lung specimen taken at the height of lipemia and a second taken later when the lipemia had cleared, and there are also specimens of lung taken before oil was fed and again some hours later, though no lipemia occurred in the interval. In some of these specimens the difference in appearance is striking but not in all.

The blood specimens were oxalated, centrifuged in an angle centrifuge at about 4000 r.p.m. for 10 min., and the plasma appearance noted. Measurements of opacity were made with a Coleman Junior Spectrophotometer at a wave length of 680 mμ. Many plasmas showed a slight though distinct fogginess to reflected light but were clear to transmitted light. It has been suggested by Moreton (6) that this phenomenon may be due to very minute chylomicrons. The simple measure of light transmittance through the plasma serves very well as an index of the degree of lipemia in the more clearcut examples, though it is not a completely satisfactory procedure in very minimal lipemias. Many of the plasmas were also examined microscopically, under the 40 X objective with a 15 X eyepiece and with dark field illumination, and a rough chylomicron estimation was made (5). The lung specimens for section were dropped into 5% formalin saline for 24 hr. or more. Frozen sections were cut, stained with Sudan IV and haematoxylin, and mounted in Farrent's solution. Chemical determination of total lipid was also done on many specimens. From several animals other tissues were also taken. In the liver there is a patchy fatty metamorphosis, oil droplets of variable size, both intracellular and interstitial, being present during the fat absorption. Other tissues have not yet been studied in detail.

It was thought that the conditions found were not caused by faulty histological technique, because standard methods had been used by competent workers; however the effect of variations in fixing and freezing has been examined. Satisfactory sections were not obtained from fresh lung but no difference could be seen between blocks fixed for about 18 hr. and those fixed

for a week or more.   Blocks frozen and thawed several times also gave the same appearance as those frozen only once.   As a further check on the possibility of the freezing causing a change in dispersion of the lipid, gelatin to the extent of 5% was added to lipemic plasma and sections were cut of this gel, at once and after fixation in 5% formol saline for varying periods. In all cases the appearance was alike and consisted of a very finely granular Sudan staining material.   The suspension was somewhat coarser but similar in appearance to the chylomicrons of the original plasma, and there was no suggestion of formation of oil globules.   A variant of procedure that did make a difference to the appearance of the sections of lung was to allow them to dry on the slide, after staining before mounting.   Oil globules in these sections seemed to lose their refractility and to be somewhat absorbed by the surrounding tissue; they were fainter and more diffuse.   Care was therefore taken to add Farrent's solution for mounting before the sections became dry.

From these observations therefore, it would seem that the presence of the oil globules in the tissues is real, and not due to histological artefacts.

The earlier consistent and striking difference between the lung appearance in the lipemic and fasting animals has not been maintained.   Occasional oil globules are quite commonly seen in the normal controls and many of the lipemic animals failed to show a clear-cut increase in the number or size of oil globules.   However it remains true that the only preparations showing masses of oil were from animals that had been fed oil.   Of the 14 normal controls, six were rabbits, four were guinea pigs, two were cats and there was one dog and one monkey.   All were included in the control group; there seemed to be no species difference in the number or size of oil globules.   Two of the controls showed moderate oil while 12 showed minimal or none.   Of the 38 test animals, 15 showed marked oil deposits, six moderate, and 17 minimal or none.   The controls were not fed on the day of the experiment, but had been fed as usual

Frozen sections stained with Sudan IV and haematoxylin.

The red staining oil globules show as black on the orthochromatic film, but other dense areas may also be very dark.   Some of the definite red oil globules are marked with white crosses.

The magnifications given are those of the microscope objective and ocular that were used.

The total enlargement of the final printed figure is about one-half this.

FIG. 1.   *Cat No. 17, 7 cc. of cod-liver oil by mouth, lung taken three hours later, mild lipemia. Lung, ×40 ×7.*

FIG. 2.   *Dog No. 17, 60 cc. of cod-liver oil mixed with food, lung taken five hours later, marked lipemia.   Lung, ×40 ×15.*

FIG. 3.   *Cat No. 12, 10 cc. of cod-liver oil by mouth, lung taken six hours later, marked lipemia.   Lung, ×40 ×7.*

FIG. 4.   *Dog No. 8, 80 cc. of cod-liver oil injected intravenously at the rate of 2 cc. per minute.   Lung taken at once.   No lipemia.   Lung, ×40 ×7.*

FIG. 5.   *Guinea pig No. 15, 2 cc. of cod-liver oil by mouth, lung taken five hours later, marked lipemia.   Lung, ×40 ×7.*

FIG. 6.   *Guinea pig No. 28, 1.5 cc. of cod-liver oil by tracheal cannula, lung taken 15 min. later.   Animal under sodium pentobarbital.   Lung, ×10 ×7.*

PLATE I
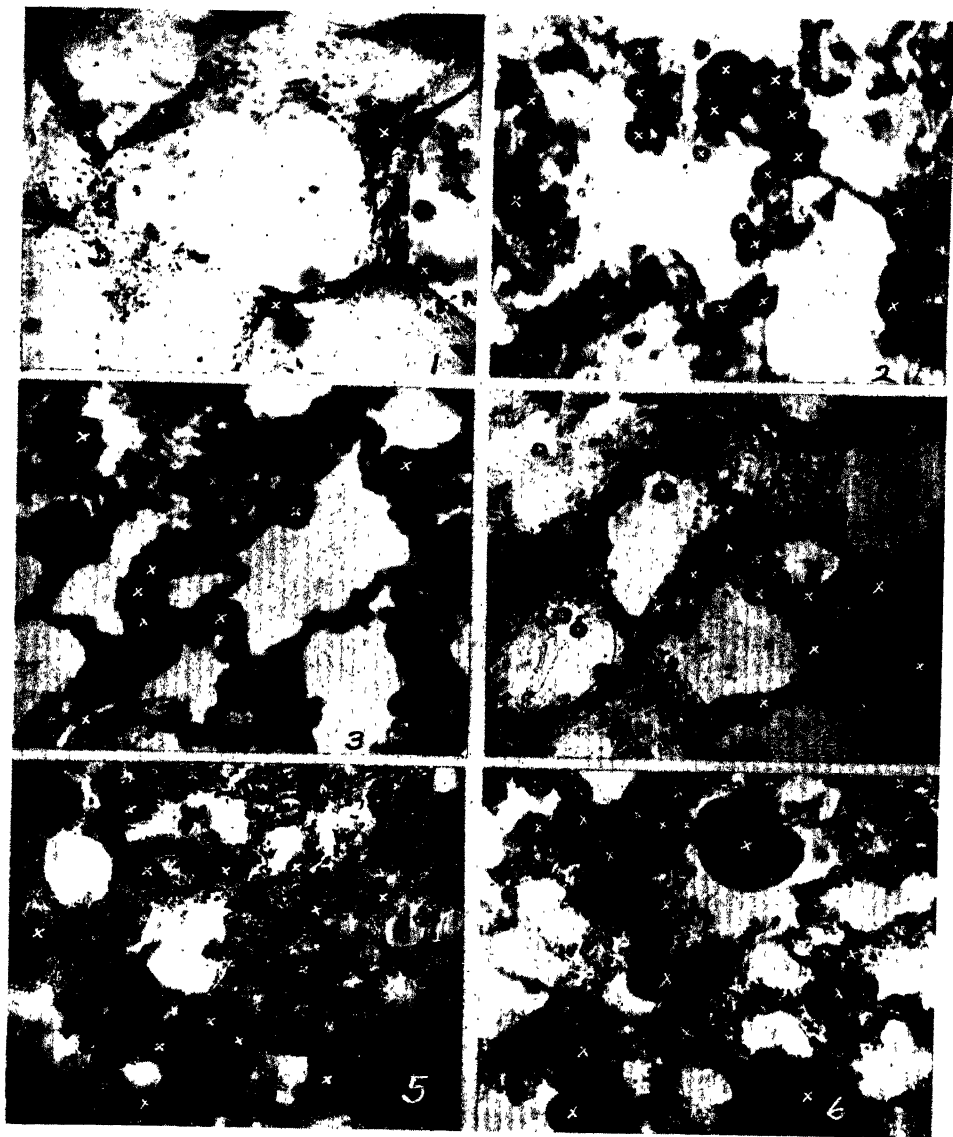
on the previous morning. Contrasting the normal and test groups in the marked oil and minimal oil categories, the number of animals in each group is shown in Table I below.

TABLE I

NUMBER OF ANIMALS SHOWING OIL DEPOSITS IN LUNG

	Marked	Minimal
Normal, not fed	0	12
Oil fed	15	17

These results show a highly significant difference between the lung oil deposits of the normal and oil fed groups, the figures yielding a corrected Chi square of 6.6 (Yate's correction).

The results for all the animals, grouped in various ways, are shown in Table II.

TABLE II

NUMBER OF ANIMALS SHOWING OIL DEPOSITS IN LUNG

	Marked	Moderate	Minimal
Normal—fasting	0	2	12
Oil fed—all species	15	6	17
" —non lipemic, all species	5	2	6
" —lipemic, all species	10	4	11
" —cats	6	3	1
" —dogs	3	2	7
" —guinea pigs	5	1	8
" —rabbits	0	0	1
" —monkey	1	0	0

It will be noted from these figures that there is no difference in the oil fed group between the animals developing lipemia and those not developing lipemia. As between species, the cats seem to show greater oil deposits after feeding but the difference is hardly significant. Taking the group as a whole, however, it is evident that there is a definite positive relationship between oil feeding and interstitial oil globules in the lungs.

Furthermore, in many of the sections there were cells containing Sudan staining granules. They were particularly numerous just under the pleural surface. These intracellular lipid granules seemed to be more numerous in the oil fed animals and, in these, in a number of areas they appeared to be coalescing to form definite oil globules that then were extruded to form intra-capillary globules. Whether or not some such mechanism is the source of many of the interstitial oil globules is purely speculative. Moreton (6) has

suggested that phagocytosis is stimulated by larger rather than by the smaller chylomicrons and has pointed out that in alimentary lipemia the chylomicrons become larger, a greater number thus becoming visible.   On the other hand, the oil globules in lung appear in the animals fed fat whether or not they develop lipemia.   According to Frazer (3) the lipemia is due to lymphatic fat absorption while active fatty acid absorption without a lipemia may occur by the portal route.   The finding of excess lung oil in oil fed animals not exhibiting lipemia coupled with the marked fatty changes in the liver suggest that the liver may be the source of at least some of the oil globules found in the lung.

Perfusion Experiments

Because of the uncertainties involved in the production of alimentary lipemia and the practical difficulties in obtaining animals in a comparable degree and stage of lipemia, perfusion experiments were devised.   It was thought that a lipemic plasma from one animal perfused through the lung of the same or another animal at a controlled rate would allow more uniform experimental conditions than was possible when simple feeding experiments were used. This was achieved only a few times.   Difficulty was experienced in obtaining enough lipemic plasma from small animals, and the expedient of perfusing dog plasma through rabbit lung was tried.   As the perfusion lasted only about one-half to one hour and the animals were not previously sensitized to the foreign protein, it was thought that it would be satisfactory.   The animals were anesthetized with sodium pentobarbital, given artificial respiration by tracheal cannula and pump, and the thorax opened. Cannulae were rapidly inserted into the pulmonary artery and left auricle, and plasma (which usually showed a light transmittance of about 50%) run into the artery by a continuous injection pump at the rate of about 5 cc. per minute.   The effluent from the left auricle was collected serially in centrifuge tubes.   At the beginning of the perfusion there was often a slight reduction in the opacity of lipemic plasma during its passage through the lung, but in the main it passed through unchanged.   After the perfusion the lungs showed no excess oil globules.

These experiments suggest that the lung does not take up any great amount of oil from lipemic plasma but they are not conclusive.   The rate of the perfusion is probably very much less than the blood flow of the intact animal. The total volume of fluid perfused and its fat content may well be too small to allow a demonstrable amount of oil to collect in the lung, even if it were doing so.

To overcome this difficulty, an attempt was made to make an artificial oil emulsion, which could be made much more concentrated and in large quantities.   Cod liver oil or olive oil emulsions were successfully prepared with a particle size of less than 1μ, and a transmittance of perhaps 1.0%.   These emulsions were quite stable and kept well for days and, usually diluted to give a transmittance of about 10%, were perfused in a large number of rabbits and cats at the rate of 1 to 5 cc. per minute.   They were prepared as follows,

the emulsifying agents being suggested by Frazer (4): 5 to 10 cc. oil, 2 to 3 cc. monobutyrin, 1 to 2 cc. oleic acid, 0.1 gm. sodium glycocholate, 3 to 5 gm. isinglass, and sometimes a trace of lecithin were made up to 100 cc. with Locke's solution, mixed, and brought to a pH of about 7.2. This was then passed through a hand homogenizer and centrifuged vigorously, the subnatant fluid being carefully drawn off and examined microscopically. If any globules larger than minute particles exhibiting Brownian movement were seen, the emulsion was recentrifuged. Some batches emulsified easily and others did not work at all. The protein was essential.

Even though the emulsion seemed stable and could be diluted freely with saline, the perfusion experiments with artificial emulsions were a failure. During passage through the lung the emulsion broke and the effluent was curdled. The lung showed masses of debris and oil globules, sometimes typical of pulmonary fat embolism. It was then found that the mere addition of blood or plasma to the artificial emulsion caused the emulsion to break. Attempts to use plasma instead of the isinglass in the original preparation of the emulsion proved unsuccessful and the method was dropped.

Lung Fat Determinations

Some of the lungs were not only examined histologically for oil but chemically as well. Portions of wet lung (1 to 2 gm.) were digested with sodium hydroxide over a steam bath. The digest was neutralized, extracted with ether and alcohol-ether, and the ether layer evaporated to dryness by radiant heat. The residuum was then extracted with petroleum ether, rinsed into weighed beakers, dried with radiant heat, and weighed. Duplicate samples checked within about 10%. The lipid content of the lungs of dogs, cats, and rabbits ranged from 11.0 mgm. to 22.4 mgm. per gm. of wet lung, the mean being 16.0 mgm. The standard error of the mean was ±0.92. In guinea pigs the value was significantly higher, ranging from 20.5 to 29.8, averaging 24.4 mgm. (standard error ±1.21). In these animals more Sudan staining intracellular granules were found than in the other species. In no species was there a significant relation between the degree of lipemia and the lipid value. Microscopically there was often a peribronchial accumulation of fat, probably adipose tissue, that was very variable and it was thought that this accounted for most of the lipid found. Specimens frankly loaded with oil gave high values, but, except when oil was aspirated, the values were no higher than in some normal specimens practically free of interstitial oil globules.

Aspirated Oil

The animals were not anesthetized and the oil was given by mouth; it was assumed that unless some choking occurred during the feeding of oil no aspiration of oil would have taken place.

It has been noted that some animals (guinea pigs) exhibited some choking when the oil was being given by mouth. It was stated that these animals were discarded. The lungs of five, however, were examined though the results have not been included in previous discussion. Three of these five animals showed considerable oil in large globules in the lung, most of the globules being in the alveolar spaces. This finding makes it necessary to consider the possibility that oil was aspirated by the animals that did not choke. Cannon (1), in a review of lipid pneumonia has emphasized the possibility that prolonged administration of oil, particularly by the nasal route but also by mouth, may result in aspiration oil pneumonia.

Two dogs were given their oil soaked into biscuits, and two cats were given theirs in a fish paste. In all four instances the food was eaten naturally and quickly, so it is extremely unlikely that any oil would be aspirated. The lungs of three of these animals showed interstitial oil globules but not definitely more than sometimes found in normal animals. The lung from one dog, however, showed many interstitial globules (Fig. 2), and many cells contained Sudan staining granules some of which seemed to be coalescing into globules that were extruding into the tissue spaces.

A number of animals were then given intratracheal oil. All were anesthetized with sodium pentobarbital and all were breathing naturally. A guinea pig had one lung removed as control and then 1.5 cc. cod liver oil was dropped into the tracheal cannula. Fifteen minutes later, in contrast to the clear control, the test lung contained masses of oil globules that filled many alveoli (Fig. 6). By analysis the lung contained nearly three times as much lipid as the control. Note that the photomicrograph was made with the low power objective; the intra-alveolar oil droplets are much larger than those in Fig. 5, which is also from a guinea pig. This animal had been given oil by mouth, without choking, and practically no oil droplets were found in the lung. However in one small area much oil was found, and as seen by the figure, it is intra-alveolar. Presumably, some aspiration had occurred. In this case the oil was given five hours before the lung was obtained while in the case of Fig. 6, there was only 15 min. between administration of oil and the taking of the lung. This time relationship probably accounts for the difference in appearance between Fig. 5 and Fig. 6, but the intra-alveolar oil in these contrasts sharply with the intravascular or interstitial globules in Figs. 1, 2, and 3.

A rabbit was then given 1 cc. of oil by tracheal cannula. Ten minutes later one lung was removed. This lung was clear of oil. The other lung taken after one and one-half hours was loaded with oil in the alveolar spaces, and its lipid content by analysis was more than three times that of the first. There was no lipemia. Two litter mate kittens were then used, one as control. The other was given two drops of oil by tracheal cannula and lungs were taken after one hour and three hours. Neither of these nor the control showed excess oil by microscope, nor did lipid analyses reveal any differences. Two rabbits and a guinea pig were then allowed to inhale an oil fog for about an

hour. The oil with 0.1% Aerosol T and an equal volume of water was nebulized and the fog directed into the side tube of a tracheal cannula. None of these three lungs showed excess oil.

From these experiments it seems clear that small amounts of oil can enter the trachea without reaching the alveoli. Large quantities of oil placed in the trachea do flood the lung, after some delay. When this does occur, however, the oil globules are primarily in the alveoli and the appearance of the specimens is quite different from that described in relation to alimentary fat absorption. In the latter cases the oil is chiefly intravascular or interstitial and the number of globules found in the alveoli is much less. In some areas the appearance resembles that of definite pulmonary fat embolism produced by intravenous injection of oil. Fig. 4 is lung from a dog into which 80 cc. of cod-liver oil was injected intravenously at a rate of 2 cc. per minute. It will be noted that large amounts of oil (Fig. 6) are found to be intravascular but many globules are also found in the alveoli; the appearance is similar to that described by Warren (7) as pulmonary fat embolism.

Though the above evidence lends strong probability to the belief that in our oil feeding experiments the appearance of the lung was not due to oil aspiration, it is not quite conclusive. The direct approach to the problem would be to ligate the oesophagus, or to block entry of the trachea by a tracheal cannula, and inject the oil directly into the stomach. It was thought necessary to try this method even though it was feared that the operative procedure would delay or even stop oil absorption.

Under sodium pentobarbital anesthesia the oesophagus of four cats was cut and both ends closed, the oil then being injected into the lower portion of the ligated oesophagus. A tracheal cannula was placed in one cat and two puppies and the oil given by stomach tube. Lung was taken two to five hours later. In only one animal was there a lipemia and in this animal the lung findings were indefinite. Three animals showed no signs of oil absorption (intestinal quiescence, invisible lymphatics, oil still in stomach at autopsy) and in these the lungs were negative for oil droplets. The other three animals showed some indication of alimentary activity (visible chyle in lymphatics, or intestinal movements, or little oil in stomach) and in all of these the lungs contained interstitial or intravascular oil globules of size and number not seen in the other animals.

Thus the lung findings discussed may occur when there can have been no aspiration of oil; therefore it is reasonable to conclude that they are not due to oil aspiration.

Conclusions

Frozen sections of lung from dogs, cats, guinea pigs, and monkeys stained with Sudan IV and haematoxylin sometimes reveal intracellular Sudan staining granules and interstitial or intravascular oil globules. These are more numerous in animals absorbing a fatty meal and in a considerable number of these, patchy areas of oil are found that resemble pulmonary fat embolism.

The appearance is not due to a histological artefact nor is it due to the aspiration of oil.

Lung fat determinations give values higher than the normal range only when excessive oil deposits are present.   Guinea pigs give higher values than cats, dogs, and rabbits.

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THE EFFECT OF BARBITURATES AND OTHER SUBSTANCES ON MOTION SICKNESS IN DOGS[1]

By R. L. Noble[2]

Abstract

A large series of barbiturates and other substances have been tested for their ability to prevent motion sickness in dogs. Many compounds were found to possess this activity, which was not related to the anesthetic property of the compound. A number of barbiturates were found to be considerably more active than V-12, which was used as a standard. Pyridoxine, hyoscine, and streptomycin were inactive in the tests described. Bulbocapnine possessed half the potency of V-12 and showed an additive effect when the two substances were given together.

In a previous paper (Noble (2)) a detailed study of the effectiveness of various types of motion in inducing vomiting in dogs was reported. A method of assaying motion sickness preventives on dogs was also described (Noble (3)). In a search for an effective form of human therapy a large number of barbiturates and other substances were tested on dogs. Many of these proved to have the ability to prevent motion sickness to a greater or lesser extent and not cause any undesirable symptoms in the animal. Since the effect on motion sickness appears to be a specific property of the compound and not related to its hypnotic action it was essential to predetermine what dose would be tolerated by dogs without causing sleepiness, in-co-ordination, or anesthesia. Following this the substance was administered to susceptible animals that were then exposed to motion on a swing. Since the number of compounds to be tested was large, it has not been possible to determine potency in a quantitative manner except for particularly interesting substances. For practical reasons only the effects of oral administration has been studied. It should be stressed therefore that where compounds are listed as inactive the statement applies only to the dose that was used.

Methods

A colony of dogs susceptible to motion was established and swung in a mechanical swing at regular intervals as previously described (Noble (2)). The animals were fasted for 18 hr. before the test, the drug was administered in a small portion of minced meat two to four hours before swinging except in special cases where indicated. Immediately before being placed on the swing the animal was fed a moderate meal of meat. When dogs of different degrees

of susceptibility were used, these were divided by their response to different intensities of motion as reported in a previous paper (Noble (2)). Vomiting was used as the only criterion of sickness but in some cases where the time taken to vomit was prolonged to 40 min. and much beyond the control time, the animals were listed as "improved" by treatment. Swinging was discontinued immediately after the animal vomited or after 45 min., in which case the animal was listed as protected. The barbituric acid derivatives were prepared and supplied through the co-operation of Abbott Laboratories and Eli Lilly and Co. Compounds supplied by the latter are designated by the letter A following the number. In initial tests each substance was given to groups of five to eight susceptible dogs. It was soon noted however that the effectiveness of therapy varied widely and was related to the degree of susceptibility to motion of the animal. Since it was possible to determine the susceptibility of the animals they were divided into three groups. In a second series of tests the susceptible dogs were selected at random but the dose of the compound was altered depending on whether the animal was of low, medium, or high susceptibility (Noble (2)). The group of dogs of medium susceptibility were considered as average and given the particular dose. The low susceptibility group received one-half this dose and the highly susceptible group, twice the dose. In the final series of tests the most satisfactory procedure was adopted so that groups of dogs of the same degree of susceptibility were selected and their response determined to one compound, V-12, which served as a standard. Other substances were then compared with V-12. The results of these three types of tests will be listed separately in results.

Results

Substances of No or Low Protective Action

For preliminary screening many substances were tested on two to four dogs (or more where indicated). If there was no evidence of protection in the dose used the substances were not considered worthy of further study. For reference purposes they are listed in Table I.

It is of interest to note that belladonna derivatives appear to be inactive in dogs in contrast to their protective action in humans. Similarly the vitamins, nicotinic acid and pyridoxine were also inactive.

Compounds Tested on Susceptible Dogs Selected at Random

A series of compounds were administered at various dose levels to groups of susceptible dogs. These have been listed in Table II in apparent order of activity although the results are of little quantitative value. The first figure in the table refers to the number of dogs protected against vomiting, the second to those that went 40 min. or more and then vomited but were considered improved, and the last figure to those unaffected by treatment.

TABLE I

Substances Inactive in Protecting Against Motion Sickness in Dogs

Compound number	Substances*
Dose, 100 mgm./kgm.	
1	N-Methyl b.a.
3	C-C-dimethyl b.a.
19	Allyl-1-methylbutylacetylurea
1-A	Thiourea
8	*n*-Butyl-*n*-amyl b.a.
Dose, 30 mgm./kgm.	
7	Ethylacetoaminophenyl b.a.
11	*n*-Butylcyclohexyl b.a.
16	Diallyl-N-allyl b.a. (calcium salt)
28	Isopropylcyclohexenyl t.b.a.
37	Ethyllauryl t.b.a.
38	Ethyloctyl t.b.a.
39	Ethylheptyl t.b.a.
40	Ethyldecyl t.b.a.
42	Dibutyl t.b.a.
43	Allylbenzyl t.b.a.
49	Monobenzyl t.b.a.
61	Diphenylenehydantoin
63	Allylphenyl t.b.a.
65	*n*-Butylphenyl t.b.a.
69	*n*-Hexyl-*n*-propyl t.b.a.
77	Ethylphenyl t.b.a.
54	Mono-1-3-dimethylbutyl t.b.a.
26	Ethyl-1-methylbutylimino t.b.a.
Dose, 10 to 20 mgm./kgm.	
51	Ethylcyclohexenyl t.b.a.
72	Ethylisopropyl t.b.a.
73	Ethyl-2-ethylbutyl t.b.a.
86	Ethyl(methylamylcarbinyl) t.b.a.
11-A	*n*-Propylcyclopentenyl t.b.a. (sodium salt)
9	Ethyl-2-ethylbutyl b.a.
15	Allylbutylimino t.b.a.
2-A	Diphenylhydantoin
29	Allyl-1-methylbutylimino t.b.a.
47	Ethylcyclohexyl t.b.a.
Dose, 5 mgm./kgm.	
18	Ethyl-*sec*-butyl-N-methyl b.a. (calcium salt)
58	Ethyl(methylvinylcarbinyl) b.a.
59	Ethyl-2-pentenyl b.a.
10	Ethyl-1-methylbutyl-N-methyl b.a. (calcium salt)

* *Dosage of additional substances*
    "Vasano" (hyoscine camphorate), 0.1 mgm.;
    hyoscyamine camphorate, 0.4 mgm.;
    nicotinic acid 25 mgm. and 50 mgm. (six tests);
    pyridoxine 50 mgm. and 100 mgm. (eight tests).
Note: *b.a.* = *barbituric acid; t.b.a.* = *thiobarbituric acid.*

TABLE II

PROTECTIVE EFFECT OF BARBITURATES ON SUSCEPTIBLE DOGS

Compound number	Substance	Dose, mgm./kgm.			
		30	20	10	5
5	Allyl-*sec*-butyl b.a.			1–0–0	3–1–0*
9-A	Ethylcrotyl t.b.a. (sodium salt)			4–0–0	1–1–1
91	Isopropyl-1-methylallyl t.b.a.			3–0–0	0–1–0
104	Allylisoamyl b.a.		3–0–0	4–1–0	
44(V-12)	Ethyl-β-methylallyl t.b.a.		4–0–0	5–2–1	1–0–2
95	Ethyl-β-methylallyl t.b.a. (sodium salt)			1–1–0	1–0–3
100	Ethyl-β-methylallyl t.b.a. (calcium salt)				0–2–0
108	Allylcrotyl t.b.a.			2–2–0	0–3–0
64	Ethyl-1-methylallyl t.b.a.			4–1–3	
87	Allyl-1-methylisoamyl t.b.a.			3–4–0	
81	Allyl-1-methylbutyl t.b.a.		1–0–1	3–2–0	0–1–1
71	Allyl-*sec*-butyl t.b.a.			3–0–2	0–1–1
50	Allylisopropyl t.b.a.			2–2–3	
12	Ethylallyl t.b.a.	8–2–1		2–0–3	
23	Ethyl-*n*-butyl b.a.	3–0–0	4–0–0		0–0–2
24	Ethyl-1-methylbutyl b.a.	2–0–1	4–1–1		1–0–6
35	Ethyl-1-methylbutyl t.b.a.	3–0–0	5–0–1	0–0–2	
45	Ethyl(γ-chlor-β,γ-butenyl) t.b.a.	1–0–0	4–0–0	2–0–3	
55	Ethyl-2,4-dimethylpentyl b.a.		3–1–0	1–1–2	
33	Ethyl-*sec*-butyl-N-methyl b.a. (calcium salt)		3–0–1	0–2–2	
75	Ethyl-*n*-amyl t.b.a.		3–0–0	0–3–1	
36	Ethylisoamyl t.b.a.	6–1–1	4–0–1		
46	Ethylbutyl t.b.a.		3–0–2	2–2–0	
48	Methyl-*sec*-butyl t.b.a.		3–2–1		
105	Ethyl-2-ethylhexyl b.a.		3–1–2	1–1–2	
4-A	Ethylphenyl b.a. (sodium salt)	1–0–1	1–0–1		
5-A	Diethyl b.a. (sodium salt)	1–0–2	2–0–2		
84	Allyl-*n*-amyl t.b.a.		3–0–3		
57	Diallyl-N-allyl b.a.		2–1–0		
62	*sec*-Butylcrotyl t.b.a.	1–1–2	2–0–0		
6	Ethylmethylhexylcarbinyl b.a. (sodium salt)	3–0–0	1–0–1		
56	Ethyl-*n*-hexyl t.b.a.	7–2–2			
93	Ethyl-*n*-hexyl t.b.a. (sodium salt)			1–1–1	0–0–3
93-B	Ethyl-*n*-hexyl t.b.a. (calcium salt)	0–1–4			
31	Diallyl t.b.a.	7–0–1			
7-A	*n*-Butyl-1-methylallyl t.b.a.	5–3–0		0–0–3	
67	Allyl-*n*-hexyl t.b.a.	2–3–1			
68	*n*-Hexyl-β-methylallyl t.b.a.	2–3–0			
32	Methyl-*sec*-butyl-N-methyl b.a. (sodium salt)	3–1–2			

* *Number of dogs under each dose divided to show number protected, improved, and negative.*
Note: *b.a.* = *barbituric acid; t.b.a.* = *thiobarbituric acid.*

TABLE II—*Concluded*

PROTECTIVE EFFECT OF BARBITURATES ON SUSCEPTIBLE DOGS—*Concluded*

Compound number	Substance	Dose, mgm./kgm.			
		30	20	10	5
21	Ethyl-β, β-dimethoxypropyl b.a.	1–1–4			
22	Ethyl-α, γ-dimethoxypropyl b.a.	0–3–0			
41	Ethylcyclopentyl t.b.a.		1–1–0		
52	Ethyl-*sec*-butyl t.b.a.			1–1–0	
74	Diethyl t.b.a.	1–1–0	0–1–1		
85	Ethyl(methylhexylcarbinyl) t.b.a.		1–0–0	0–1–1	
99	Allyl-*sec*-butyl b.a. (sodium salt)			1–1–0	
92	*n*-Propyl-1-methylallyl t.b.a.		2–3–2		
103	*n*-Propylcrotyl t.b.a.		1–1–2	0–1–3	
78	*sec*-Butylallylimino t.b.a.	0–0–1	0–1–6	1–0–3	
79	*sec*-Butyl-β-methylallylimino t.b.a.		0–0–3	0–0–1	
83	Allyl-2-ethylbutyl t.b.a.		0–0–1	3–1–1	
88	Allyl-*n*-butyl t.b.a.		0–1–1		
·102	Ethylcrotyl t.b.a.				1–0–1
101	Ethylcrotyl t.b.a. (sodium salt)				1–1–0
		Dose per kgm.			
		100	90	80	50
53	Mono-1-methylbutyl t.b.a.				2–1–5
27	Ethylphenyl t.b.a.	3–0–0	0–0–2		
34	Ethyl-1-methylbutylacetamide	3–0–0		1–0–2	

* *Number of dogs under each dose divided to show number protected, improved, and negative.*
Note: *b.a.* = *barbituric acid; t.b.a.* = *thiobarbituric acid.*

It may be seen that the majority of these compounds exhibited the property of protecting dogs against motion sickness and not causing any unfavorable symptoms. Because of the wide variation in susceptibility of the animals and the small numbers used in each test, the results can only be considered as roughly quantitative. It is of interest to note that in some cases the sodium or calcium salts seemed of less activity than the acid. This and other observations indicate that the more insoluble acid forms of the barbiturates are the most effective in therapy.

Compounds Tested When the Dose was Adjusted According to Susceptibility of the Dogs to Motion

As previously mentioned a second series of tests were made in which the animals tested received varied doses of the drug depending on their previously determined susceptibility to motion. The dose that is listed in the table refers to that given to the dogs of moderate susceptibility, animals in the

group of low susceptibility would therefore receive half this dose and those of high susceptibility twice this dose. The substances are arranged in apparent order of activity in Table III.

TABLE III

EFFECT OF SUBSTANCES AT DOSE LEVELS PROPORTIONAL TO SUSCEPTIBILITY OF THE DOGS

Compound number	Substance	Dose, mgm./kgm.		
		10	5	3
125	Dicrotyl b.a.	4–1–1	5–2–1	3–2–1*
106	Allyl-β-methylallyl t.b.a.		3–3–0	2–4–4
104	Allylisoamyl b.a.	7–0–0	3–5–0	4–2–3
115	n-Propyl-β-methylallyl b.a.	5–1–0	3–2–2	
110	1-Methylallyl-β-methylallyl t.b.a.		3–2–2	
44	Ethyl-β-methylallyl t.b.a.		3–1–3	
124	Allyl-β-methylallyl b.a.	1–0–0	3–1–2	
107	Di-β-methylallyl t.b.a.		2–2–2	
109	Crotyl-β-methylallyl t.b.a.	1–1–0	2–2–2	
9-A	Ethylcrotyl t.b.a. (sodium salt)		3–0–3	
12	Ethylallyl t.b.a.		4–1–3	
117	sec-Butylcrotyl b.a.		3–0–3	
116	sec-Butyl-β-methylallyl b.a.		2–1–3	
113	Diallyl-N-methyl b.a.		2–0–3	
118	Ethyl-β-methylallyl b.a.	5–1–0	1–3–2	
126	Ethyl-isoamyl b.a.		2–2–7	4–1–5
24	Ethyl-1-methylbutyl b.a.		3–0–4	
122	Di-1-methylallyl t.b.a.		1–2–3	
7-A	n-Butyl-1-methylallyl t.b.a.		2–1–4	
5	Allyl-sec-butyl b.a.		1–1–4	
121	Dicrotyl t.b.a.	3–0–2	1–1–3	
123	Crotyl-1-methylallyl t.b.a.		2–0–4	
111	n-Propylallyl t.b.a.		1–1–5	
112	n-Propyl-β-methylallyl t.b.a.		1–0–5	
90	Ethyl-1-methylisoamyl t.b.a.	6–1–0		
64	Ethyl-1-methylallyl t.b.a.	5–0–2		
105	Ethyl-2-ethylhexyl b.a.	0–2–3		
114	Ethyl-β-methylallyl-N-methyl b.a.	2–1–2		
92	n-Propyl-1-methylallyl t.b.a.	4–0–3		
91	Isopropyl-1-methylallyl t.b.a.	3–0–0		
95	Ethyl-β-methylallyl t.b.a. (sodium salt)	1–1–0		
103	n-Propylcrotyl t.b.a.	0–1–2		
83	Allyl-2-ethylbutyl t.b.a.	1–0–3		
88	Allyl-n-butyl t.b.a.	0–0–3		
105	Ethyl-2-ethylhexyl b.a.	0–2–3		
60	5,5-Diphenyl-2-thiohydantoin		0–0–3	
119	3-Nitrophthalylurea	0–0–2	0–0–5	

    * *Number of dogs under each dose divided to show number protected, improved, and negative.*
Note: *b.a.* = *barbituric acid; t.b.a.* = *thiobarbituric acid.*

Compounds Compared with V-12 as Standard

In the third series of tests only dogs of high or moderate susceptibility to motion have been used since these were found to give more consistent results than those of low susceptibility. Each dog was standardized against V-12(ethyl-β-methylallylthiobarbituric acid) so that other substances were compared with this substance. Table IV shows the results obtained using

a group of four to five dogs of the highest susceptibility to motion and Table V those with a group of six or seven dogs of moderate susceptibility. The average time of vomiting of those dogs that vomited is listed in parentheses.

<div align="center">TABLE IV</div>

<div align="center">EFFECT OF SUBSTANCES COMPARED WITH V-12 ON SIX TO SEVEN
DOGS OF MODERATE SUSCEPTIBILITY</div>

Compound number	Substance	Dose, mgm./kgm.		
		10	5	2.5
		Percentage protected (and average time of vomiting)		
44 (V-12)	Ethyl-β-methylallyl t.b.a. (15 mgm./kgm. = 100%)	66 (41)	0 (18)	
137	Ethylmethylcyclopropylcarbinyl b.a.		80 (40)	60 (40)
21-A	Ethyl-1,3-dimethyl-1-butenyl b.a.			50 (28)
17-A	Ethyl-1,3-dimethylbutyl b.a.		83 (45)	42 (28)
90	Ethyl-1-methylisoamyl t.b.a.		60 (15)	
115	n-Propyl-β-methylallyl b.a.		55 (27)	0 (24)
22-A	n-Propyl-1-ethoxyethyl b.a.		40 (26)	
107	Di-β-methylallyl t.b.a.		42 (20)	
108	Allylcrotyl t.b.a.		42 (21)	
118	Ethyl-β-methylallyl b.a.		33 (22)	
133	Allyl-2-hexenyl b.a.		33 (36)	
134	Allyl-2-pentenyl b.a.		33 (12)	
26-A	Ethyl-2,4-dimethylpentyl b.a.		33 (15)	
28-A	Allyl-N-methylphenyl b.a.		20 (23)	
72	Ethylisopropyl t.b.a.		20 (30)	
75	Ethyl-n-amyl t.b.a.		20 (32)	
125	Dicrotyl b.a.	84 (32)	16 (28)	
116	sec-Butyl-β-methylallyl b.a.		15 (24)	
117	sec-Butylcrotyl b.a.			15 (28)
109	Crotyl-β-methylallyl t.b.a.		15 (28)	
123	Crotyl-1-methylallyl t.b.a.		15 (25)	
12	Ethylallyl t.b.a.	0 (25)		
126	Ethylisoamyl b.a.	100	0 (25)	
18-A	Isobutyl-1-methylallyl t.b.a.		0 (26)	
121	Dicrotyl t.b.a.		0 (21)	
103	n-Propylcrotyl t.b.a.		0 (28)	
124	Allyl-β-methylallyl b.a.		0 (32)	
104	Allylisoamyl b.a.		0 (28)	
135	Ethyl-4-pentenyl b.a.		0 (23)	
24-A	(1-Methylbutyl)ethylacetylthiourea		0 (16)	
14	Ethyl-1,3-dimethylbutyl t.b.a.			0 (24)
132	Ethyl-n-propylvinylcarbinyl b.a.			0 (26)
19-A	5,5-Dimethyl b.a.		0 (25)	
32-A	(1-Methylbutyl)ethylacetyl urea		0 (25)	

Note: *b.a.* = *barbituric acid; t.b.a.* = *thiobarbituric acid.*

TABLE V

EFFECT OF SUBSTANCES COMPARED WITH V-12 ON FOUR TO FIVE
DOGS OF HIGHEST SUSCEPTIBILITY

Compound number	Substance	Percentage protected (and average time of vomiting) Dose, mgm./kgm.		
		15	10	5
44 (V-12)	Ethyl-β-methylallyl t.b.a. (25 mgm./kgm. = 80%, 20 mgm./kgm. = 60%)	20 (12)	0 (16)	
137	Ethylmethylcyclopropyl-carbinyl b.a.			100 ⎰(2.5 mgm./kgm. = 20%)
21-A	Ethyl-1-3-dimethyl-1-butenyl b.a.			⎱(2.5 mgm./kgm. = 20%)
17-A	Ethyl-1-3-dimethylbutyl b.a.		100	25 (15)
130-X	Ethyl-n-butylvinylcarbinyl b.a.			25 (13)
131-X	Ethylmethylvinylcarbinyl b.a.		50 (13)	
28-A	Allyl-N-methylphenyl b.a.		50 (40)	
75	Ethyl-n-amyl t.b.a.		50 (35)	
22-A	n-Propyl-1-ethoxyethyl b.a.		50 (12)	
106	Allyl-β-methylallyl t.b.a.		40 (15)	
107	Di-β-methylallyl t.b.a.		40 (21)	
118	Ethyl-β-methylallyl b.a.	80 (40)	40 (21)	
115	n-Propyl-β-methylallyl b.a.		20 (26)	
109	Crotyl-β-methylallyl t.b.a.		20 (21)	
123	Crotyl-1-methylallyl t.b.a.		20 (14)	
124	Allyl-β-methylallyl b.a.		20 (30)	
27-A	Allylphenyl b.a.		20 (27)	
57	Diallyl-N-allyl b.a.		25 (29)	
70	Ethylisobutyl t.b.a.		25 (22)	
125	Dicrotyl b.a.	0 (29)		
12	Ethylallyl t.b.a.	0 (31)		
18-A	Isobutyl-1-methylallyl t.b.a.		0 (15)	
117	sec-Butylcrotyl b.a.		0 (21)	
121	Dicrotyl t.b.a.		0 (13)	
108	Allylcrotyl t.b.a.		0 (23)	
62	sec-Butylcrotyl t.b.a.		0 (18)	
54	Mono-1,3-dimethylbutyl b.a.		0 (14)	
136	5,5-Trimethylene b.a.		0 (12)	
30-A	Diphenyl b.a.		0 (10)	
26-A	Ethyl-2,4-dimethylpentyl b.a.		0 (19)	
23-A	Diethylmethylcarbinylurea	0 (16)		
25-A	n-Butyl-1-methylallyl acetamide	0 (21)		
24	Ethyl-1-methylbutyl b.a.			0 (22)
29-A	Phenylthienylhydantoin		0 (27)	
82	n-Butyl-n-propyl t.b.a.		0 (22)	
84	Allyl-n-amyl t.b.a.		0 (25)	
73	Ethyl-2-ethylbutyl t.b.a.		0 (14)	
83	Allyl-2-ethylbutyl t.b.a.		0 (13)	

Note: *b.a. = barbituric acid; t.b.a. = thiobarbituric acid.*

The results shown are considered to be the most accurate quantitative comparison of the more active compounds tested. These have been listed in terms of activity compared with V-12 in Table VI.

TABLE VI

ACTIVITY IN TERMS OF V-12

Compound number	Substance	Number of times potency of V-12	
		Highly susceptible dogs	Moderately susceptible dogs
137	Ethylmethylcyclopropylcarbinyl b.a.	6	4
21-A	Ethyl-1,3-dimethyl-1-butenyl b.a.	6	3½
17-A	Ethyl-1,3-dimethylbutyl b.a.	3	3
130-X	Ethyl-*n*-butylvinylcarbinyl b.a.	3	—
131-X	Ethylmethylvinylcarbinyl b.a.	1½–2	—
90	Ethyl-1-methylisoamyl t.b.a.	—	2
28-A	Allyl-N-methylphenyl b.a.	1½–2	1–1½
75	Ethyl-*n*-amyl t.b.a.	1½–2	1–1½
22-A	*n*-Propyl-1-ethoxyethyl b.a.	1½–2	1½–2
106	Allyl-β-methylallyl t.b.a.	1½–2	—
107	Di-β-methylallyl t.b.a.	1½–2	1½–2
108	Allylcrotyl t.b.a.	—	1½–2
118	Ethyl-β-methylallyl b.a.	1½–2	1–1½
115	*n*-Propyl-β-methylallyl b.a.	1–1½	2
109	Crotyl-β-methylallyl t.b.a.	1–1½	1–1½
123	Crotyl-1-methylallyl t.b.a.	1–1½	1–1½
124	Allyl-β-methylallyl b.a.	1–1½	1
133	Allyl-2-hexenyl b.a.	—	1–1½
134	Allyl-2-pentenyl b.a.	—	1–1½
26-A	Ethyl-2,4-dimethylpentyl b.a.	—	1–1½

Note: *b.a. = barbituric; t.b.a. = thiobarbituric acid.*

It may be seen that the activity of most compounds was of approximately the same order irrespective of the group of dogs in which they were tested. The first four compounds listed seemed to have considerably greater activity than the standard V-12.

Other Observations
Common Barbiturates

In an early experiment susceptible dogs were selected at random and tested with four of the more common barbiturates at intervals of one week. The dose used was the same in each case, 20 mgm. per kgm. body weight. The results were as follows:

Sodium neonal (ethyl-*n*-butylbarbiturate)   seven dogs protected

Sodium nembutal (ethyl-1-methylbutyl barbiturate) five dogs protected

Sodium phenobarbital (ethyl-phenylbarbiturate)   four dogs protected

Sodium barbital (diethylbarbiturate)   three dogs protected

Duration of Action

Experiments have been conducted on eight different barbiturates in which the duration of protective action has been determined. In general it may be stated that the protection of an effective dose usually persists for 18 to 24 hr. at which time the animals are either immune or considerably

improved.　Longer protection is not found unless the dose given is excessive. Using V-12, six dogs received the minimum effective dose (M.E.D.) to prevent vomiting and were swung 22 hr. later; 16% were protected.　When the dose was increased to twice the M.E.D. 66% were protected after the same interval. The same number of animals received three times their M.E.D. and were swung 48 hr. later.　In this case no dogs were protected.

Repeated Doses

If small doses of active barbiturates are given at short intervals their effectiveness does not appear to be enhanced but equal to the total dose given. Even when the doses are spaced at 10 hour intervals the same additive action is observed.　Daily doses of V-12 did not give rise to cumulative effects in that noneffective doses, even though repeated daily for 10 days, did not lead to a protective action.

Mixtures

Since it seemed possible that the drug bulbocapnine, which had been shown to be effective in preventing motion sickness in a number of dogs (Babkin, Dworkin, and Schachter (1)), might act in a different manner than barbiturates, some tests with mixtures were made.　These have been summarized in Table VII.

TABLE VII

EFFECT OF V-12 AND BULBOCAPNINE ON MOTION SICKNESS

Drug	Dose, mgm./kgm.	Protected, %	Dose, mgm./kgm.	Protected, %
Moderately susceptible dogs (five animals)				
V-12	5	0		
V-12	10	60		
Bulbocapnine	20	60		
Bulbocapnine	10			
Hyoscine	0.43 mgm. (total dose)	0		
Bulbocapnine	5	40		
V-12	5			
Highly susceptible dogs (two groups of four animals)				
V-12	15	0	15	0
V-12	20	—	20	75
Bulbocapnine	10	0	—	—
Bulbocapnine	15	0	15	0
Bulbocapnine	20	0	—	—
Bulbocapnine	10	50	10	25
V-12	15		10	
Bulbocapnine	15	50	10	50
V-12	10		15	
Bulbocapnine	15	75	15	100
V-12	15		15	

It may be seen that bulbocapnine is approximately of half the activity of V-12 when given alone. When the two drugs are given together, however, definite summation of activity occurs so that noneffective doses of each may combine to give protection.

Streptomycin

The effect of streptomycin on interference with vestibular function in humans suggested it might be effective in motion sickness. Two dogs each received a single injection of 550 mgm. of streptomycin* but no protection against motion was observed. Two other animals received 300 mgm. by injection daily for 16 days. These showed no signs of ataxia and were not protected against motion by such treatment.

Discussion

The results that have been presented are a summary of experiments on dogs to attempt to find substances of value for the treatment of motion sickness. A large number of barbiturates have been shown to possess this action and from comparative tests it is apparent that this property is independent of the hypnotic or anesthetic potency of the compound. Such substances are orally active and appear to have a prolonged action after a single dose. It is of interest that a number of substances appear to be of considerably greater activity than V-12, a compound that has been shown to be effective against motion sickness in humans (Noble (4)). On the other hand some substances such as pyridoxine and hyoscine, that are effective in some types of vomiting in humans were of no activity in the tests on dogs. Bulbocapnine showed approximately half the activity of V-12 but the two substances when given together showed an additive effect.

The large number of barbiturates that possess activity in preventing motion sickness makes it difficult to determine whether any particular chemical configuration is more effective than another. From the tables it may be seen that many thio compounds are active although with substances like Nos. 44 and 118 (Table VI) and 24 and 35 (Table II) there is little difference in activity whether the compound contains sulphur or not. The use of thio compounds was chiefly considered as a means of reducing the duration and degree of sedative action. Similarly the use of substances with longer unsaturated side chains and substituted methyl groups were particularly effective and many in overdosage were of a convulsant rather than depressant action. The highly active compound No. 137 had powerful anesthetic properties in animals. Further studies may disclose a compound of low hypnotic activity yet highly protective against motion that would be more suitable for human therapy. Thio compounds would be less desirable because of possible toxic actions.

* *The streptomycin was kindly supplied by Ayerst, McKenna, and Harrison, Montreal, Que.*

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THE USE OF POLYVINYL ALCOHOL IN THE COLORIMETRIC DETERMINATION OF MAGNESIUM IN PLASMA OR SERUM BY MEANS OF TITAN YELLOW[1]

BY F. C. HEAGY[2]

Abstract

The colorimetric estimation of magnesium in plasma or serum by means of Titan Yellow requires an agent to stabilize the colored dye complex formed between magnesium hydroxide and Titan Yellow. Details are given of a method in which polyvinyl alcohol is used to maintain dispersion of the dye lake. A single estimation can be done on 2 cc. of serum.

The Titan Yellow method for estimating magnesium in plasma or serum is rapid, easy, and accurate (1). Serum proteins are precipitated by means of trichloracetic acid, Titan Yellow is added to the resulting water-clear filtrate, and on addition of sodium hydroxide a red magnesium hydroxide – Titan Yellow complex is formed that can be measured colorimetrically. In the method reported by Garner (1) gum ghatti was used to maintain the dye lake, but in our hands this substance was not consistently effective. In the following method polyvinyl alcohol (P.V.A.) is used as a stabilizing agent. Otherwise the method is similar to that of Garner with modifications for use with an Evelyn photoelectric colorimeter and a smaller initial sample.

Method

SOLUTIONS

Stock standard—dissolve 0.500 gm. of pure magnesium ribbon in distilled water containing a minimum of hydrochloric acid and dilute to one liter.

Working standard—4 cc. of the stock standard is diluted to 100 cc. (1 cc. of the working standard contains 0.02 mgm. of Mg).

Trichloracetic acid—10% (wt./vol.).

P.V.A. solution—1 : 1000; one gram of polyvinyl alcohol No. RH-349 (Canadian Industries Limited. Medium viscosity) is stirred into cold water and dissolved by gentle heating and stirring. The solution is then made up to 1000 cc. and stored for use.

Titan Yellow solution—0.05%; 100 mgm. dissolved in water, made to 200 cc., and filtered. The solution is stored in a brown bottle and kept out of strong light. Fresh solution is made up about every two weeks.

Sodium hydroxide—4 N.

PROCEDURE

To 2 cc. of serum in a 15 cc. centrifuge tube add 4 cc. of distilled water and 2 cc. of 10% trichloracetic acid by burette. Mix well, let stand five minutes, centrifuge for five minutes at 2500 r.p.m., and filter through a Whatman No. 44 filter paper. Reserve the filtrate. To 4 cc. of the water-clear filtrate in a colorimeter tube add 4 cc. distilled water, 1 cc. P.V.A. solution, 1.5 cc. 0.05% Titan Yellow, and 2 cc. 4 N sodium hydroxide by burette in that order, mixing well between each step.

A blank is prepared by taking 1 cc. 10% trichloracetic acid, 7 cc. distilled water, 1 cc. P.V.A. solution, 1.5 cc. 0.05% Titan Yellow, and 2 cc. 4 N sodium hydroxide.

A standard is prepared by taking 1 cc. of the working standard, 1 cc. 10% trichloracetic acid, 6 cc. distilled water, 1 cc. P.V.A. solution, 1.5 cc. 0.05% Titan Yellow, and 2 cc. 4 N sodium hydroxide.

After five minutes the solutions are compared in an Evelyn photoelectric colorimeter with the No. 520 filter. The serum magnesium concentration is calculated by reference to a calibration curve (Fig. 1) obtained by analysis of standard magnesium solutions.
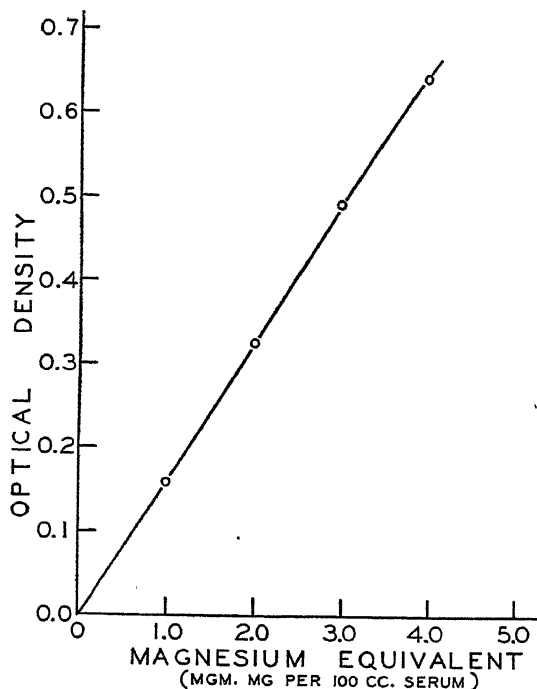

FIG. 1. *Calibration curve for magnesium estimation.*

Results

Polyvinyl alcohol consistently stabilized the dye lake.   The stabilizing effects of 1 cc. of 1 : 1000 P.V.A. and 1 cc. of 1 : 1000 gum ghatti are compared in Table I.   The same amount of magnesium is present in both tubes.   P.V.A.

TABLE I

COMPARISON OF STABILIZING EFFECT OF POLYVINYL ALCOHOL AND GUM GHATTI

Time		Optical density	
Hr.	Min.	Polyvinyl alcohol	Gum ghatti
	3	0.367	
	5		0.149
	6	0.369	
	10		0.146
	12	0.377	
	15		0.138
	22	0.367	
	30		0.137
	37	0.367	
1	16	0.367	
1	30		0.116
2	16	0.362	
3	30		0.121
5	40	0.362	
6	—		0.116
7	—	0.362	
24	—	0.372	0.152
Mean		0.367	0.134
Standard deviation		±0.0045	±0.014
Coefficient of variation		1.2	10.4

and gum ghatti are used in the respective blanks.   The coefficient of variation is 1.2 with polyvinyl alcohol and 10.4 with gum ghatti.   Gum ghatti is less effective than the table indicates because shaking the tube resuspended the precipitate of magnesium hydroxide – dye complex that settled out during the longer intervals.   Even after 24 to 48 hr., P.V.A.-stabilized solutions remain dispersed.   It is also seen in Table I that the optical density for the same concentration of magnesium with P.V.A. is more than double that with gum ghatti.   This means an increase in sensitivity and hence a smaller sample can be used.   Using a spectrophotometer it was found that P.V.A. does not shift appreciably the zone of maximum absorption by the dye lake.

Garner's paper does not indicate the effect of phosphate on magnesium estimation by Titan Yellow.   Table II shows that added phosphate in a concentration 100 times the normal serum phosphate concentration does not affect the estimation of magnesium.

In 13 estimations on pooled serum filtrates the calculated magnesium concentration was 2.56 ± 0.08 (S.d.) mgm. %.*   In 10 estimations on

*2.56 mgm. % = 2.56 mgm. of magnesium per 100 cc. of serum.

individual filtrates of a serum the estimated magnesium concentration was 2.47 ± 0.13 (S.d.) mgm. %. Recovery experiments gave an average recovery of 99% $\pm$ 3.3% (S.d.).

TABLE II

EFFECT OF ADDED PHOSPHATE ON MAGNESIUM ESTIMATION

Phosphate added (as Na_2HPO_4), mgm. % P	Magnesium estimated, mgm. % Mg
310	2.04
31	1.99
3.1	2.13
0	1.97

Note: *Magnesium present, 2.00 mgm. %. Values calculated show concentration equivalent in serum.*

Discussion

Polyvinyl alcohol effectively stabilizes the magnesium hydroxide – dye complex formed during the estimation of magnesium by means of Titan Yellow. It is preferred to gum ghatti because it is more effective as a stabilizing agent, it is a pure chemical substance, it can be prepared by simple solution rather than by leeching, and it increases the sensitivity of the method.

Acknowledgments

I wish to thank Prof. A. B. Macallum and Dr. H. A. De Luca for advice during this work.

Reference

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STUDIES ON AMOEBIASIS IN CANADA

PART II. A METHOD FOR OBTAINING VIABLE CYSTS OF *ENTAMOEBA HISTOLYTICA* FREE FROM BACTERIA[1]

By M. J. Miller[2] and W. R. Firlotte[3]

Abstract

A method for obtaining cysts of *Entamoeba histolytica* free from bacteria is described. The method involves the centrifugation and levitation of the cysts, and their passage through a series of solutions containing penicillin and streptomycin by means of a micropipette. Thousands of sterile cysts may be obtained by this method in a few hours.

Since the pioneer work of Boeck and Drbohlav (1) on the *in vitro* culture of *Entamoeba histolytica*, this parasite has been grown on artificial media with more or less success in different parts of the world. The original Locke–egg–serum medium has been enriched, simplified, and completely changed by various workers, but the fundamental growth requirements of *E. histolytica* are still unknown. Until such time as the basic factors determining and influencing the growth of *E. histolytica* are elucidated, knowledge of the disease complex, amoebiasis, will remain inadequate.

The chief obstacle to any study on the growth requirements of *E. histolytica* has been the inability to grow the parasite outside of its host in the absence of bacteria. Jacobs (2) reported the multiplication of *E. histolytica in vitro* when dead bacteria were substituted for the living organisms. However, he was unable to obtain growth after a few transfers. Recently, Shaffer *et al.* (5, 6, 7) reported on the growth of amoebae in the absence of actively multiplying bacteria and possibly in the absence of living bacterial cells. However, their medium contained practically all the products of an actively growing bacterial culture.

The task of obtaining living amoebae free from bacterial contamination, a prerequisite to any attempt to grow this parasite in the absence of bacteria, is not an easy one. The most successful method developed to date is that used by Rees and his collaborators who employed a micropipette to pick out cysts of *E. histolytica*, and subsequently freed them from bacteria by transfers through a series of sterile solutions thus gradually eliminating the bacteria by dilution. Bactericidal chemical compounds have been used with limited success by several workers. The antibiotics, penicillin and streptomycin, were used by Shaffer *et al.* (5, 6, 7) working with trophozoites in culture, but

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they found it necessary to replace the living bacteria by the undamaged products of a living bacterial culture to sustain growth, so the advantage was not a marked one.

The writers' experience with the use of a variety of bactericidal chemical agents in an attempt to rid cysts of *E. histolytica* from their accompanying bacteria has not been a happy one. It was found invariably that cysts of the amoebae were more susceptible to the chemical agent than were at least one or more species of the accompanying bacteria. The method of elimination by dilution of the bacteria accompanying cysts of *E. histolytica* by means of a micropipette as described by Rees (4) has been tested but was found very exacting and time-consuming. At present, successful use is being made of a method that involves the use of centrifugation, flotation of the cysts, immersion of the cysts in penicillin and streptomycin, and finally, dilution of the cysts in sterile solution with the aid of a micropipette. This method, which sounds complicated, is actually carried out with a minimum of effort provided a micropipette is available. Cysts of *E. histolytica* passed in stools are used in preference to cysts obtained in culture tubes mainly because the cysts in the latter are not uniformly quadrinucleate, nor are *in vitro* encystation techniques consistently successful. The method used is as follows:—

(1) The stool is broken up, suspended in tap water and sieved through a fine mesh screen (80 meshes per linear inch).

(2) The suspension of faeces and cysts in water is washed in tubes, 2.5 by 5.0 cm., for five minutes at 1500 r.p.m.; this is repeated until the supernatant fluid is clear (five to seven times).

(3) The washed sediment from all the tubes is pooled, and approximately 1 ml. amounts are placed in sterile Wassermann tubes, and sterile Ringer's solution added to nearly fill the tubes. The tubes are centrifuged for three minutes at 1500 r.p.m., and the supernatant fluid discarded.

(4) About 2 cc. of zinc sulphate solution, sp. gr. 1.100, is added to the sediment of each tube, the sediment is carefully broken up in the solution, and additional zinc sulphate solution added to within 5 mm. of the top of the tube. This is centrifuged for 90 sec. at 1500 r.p.m. The cysts, which are now floating on the surface of the zinc sulphate solution in the tube, are picked up by means of a wire loop 5 mm. in diameter and washed off into a Wassermann tube containing sterile Ringer's solution. Usually, 10 loopfuls will collect the large majority of the cysts.

(5) After the cysts from 20 or more flotations have been collected and washed off into the tube of sterile Ringer's solution, this tube is centrifuged for four minutes at 1500 r.p.m. The supernatant fluid is carefully pipetted off to within 1 cm. of the bottom, sterile Ringer's solution added, and the centrifugation repeated as above. Again the supernatant fluid is pipetted off to within 1 cm. of the bottom.

(6) The remaining fluid and cysts are transferred to a small Petri dish (10 by 60 mm.) containing 8 cu. mm. of sterile Ringer's solution to which has been added penicillin to a concentration of 500 units per ml., and streptomycin to a concentration of 1000 units per ml.

(7) The cysts, which are now free from practically all faecal debris and bacteria,* are picked up by means of the micropipette and transferred to a new Petri dish containing the antibiotics in sterile Ringer's solution. This is repeated five times although the cysts are usually sterile following the second transfer.

The micropipette has been built on the same principle as that described by Rees (4) and is used in the same manner. It is essentially a microscope frame with a mechanical stage attached. The pipette, which is made by drawing out glass tubing, is controlled by a mercury column operated through a thumb screw attached to a tuberculin syringe (Figs. 1, 2, and 3). The pipette is sterilized by redrawing as described by Rees (4). The writers have modified the basic design so that the entire pipette mechanism is mounted on the mechanical stage and moves as a unit when the pipette is moved; the danger of breaking the glass tube connecting the mercury from the syringe to the pipette is thus obviated.
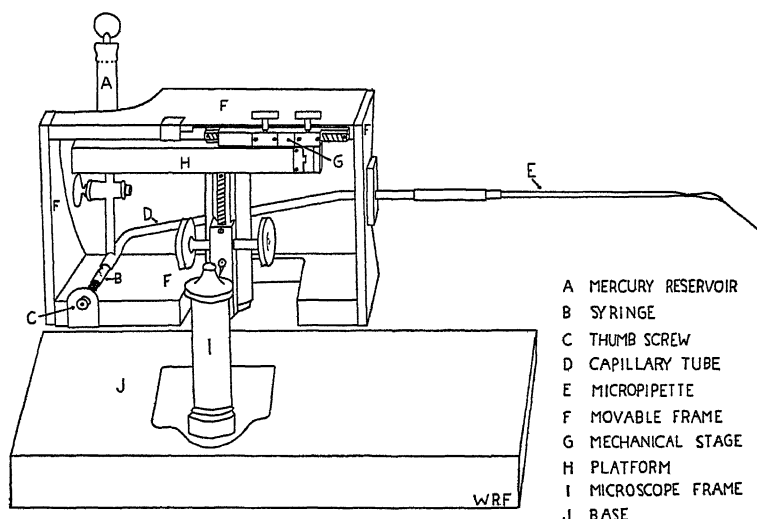

A   MERCURY RESERVOIR
B   SYRINGE
C   THUMB SCREW
D   CAPILLARY TUBE
E   MICROPIPETTE
F   MOVABLE FRAME
G   MECHANICAL STAGE
H   PLATFORM
I   MICROSCOPE FRAME
J   BASE

FIG. 1. *Diagram of micropipette to show the working parts in detail.*

Stools that show less than one *E. histolytica* cyst per low power field on simple smear will not give a heavy yield by this method. However, when necessary, the writers have used stools showing considerably less than one cyst per low power microscope field and were always sure of obtaining several

* *The examination of faecal debris and cysts at this stage for bacteria by culture methods has always revealed the presence of a species of Streptococcus, the only species apparently resistant to the antibiotics used.*

hundred sterile cysts.   On the other hand, if a stool shows a heavy infection, with five or more cysts per low power field, thousands of sterile cysts can be obtained.   In all cases, the entire procedure should not require more than eight hours' working time.   In mixed infections some difficulty may be experienced in separating *E. histolytica* cysts from those of other species. However, *Entamoeba coli* and *Endolimax nana*, which are the usual contaminants, can be differentiated in most cases by size alone.

As shown previously by one of us (3), institutions such as orphanages, etc., show a high incidence of *E. histolytica* infections in Canada, and this probably holds true for other parts of the world.   Cysts used in these experiments were obtained from stools collected in a boys' orphanage.   The well known irregularity with which *E. histolytica* cysts appear in the stool in most infections makes it necessary in many cases to obtain several consecutive stools from the same cases before a satisfactory stool is found.

The writers have used this method successfully for initiating cultures of *E. histolytica* growing with single strains of bacteria and are now studying the cultural characteristics of the amoebae growing in this manner.

To initiate cultures with sterile cysts, from 10 to several hundred cysts have been inoculated.   Routinely, six tubes are inoculated: two with 10 cysts each, two with 25 cysts each, and two with 100 cysts each.   The bacteria to be tested are added immediately after the cysts have been inoculated into the culture tubes.   To date, 10 species of bacteria have been tested, the majority of which have sustained growth of amoebae in a modified Boeck and Drbohlav (1) culture medium.   The results of these experiments will be the subject of a later report.

Acknowledgments

The photographs were made by Mr. J. B. Poole to whom the authors express their thanks.

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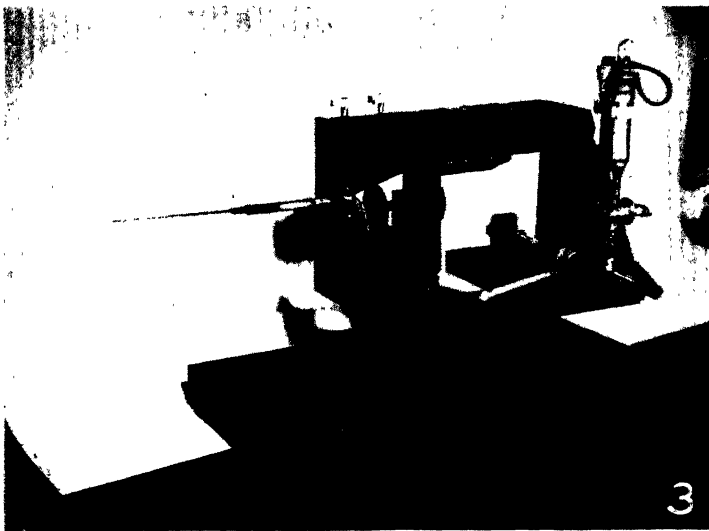7. SHAFFER, J. G., WALTON, J. G., and FRYE, W. W.   Am. J. Hygiene, 47 : 222.   1948.
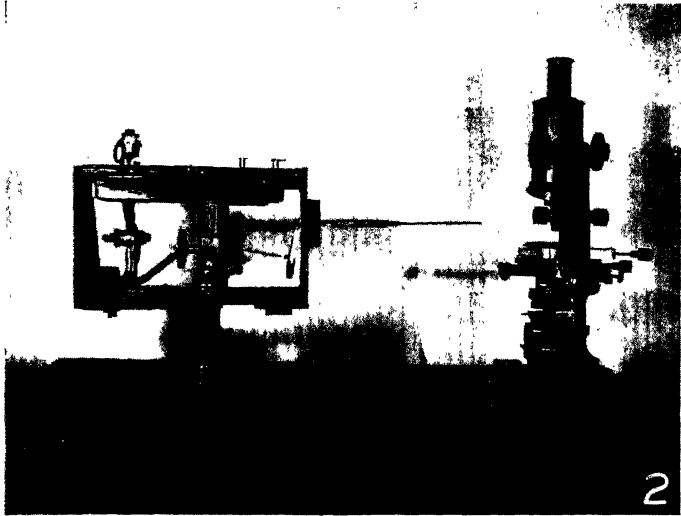
PLATE I

FIG. 2. *Micropipette set up for collecting cysts of Entamoeba histolytica.*

FIG. 3. *Micropipette viewed from behind.*

SOME EFFECTS OF THE ADMINISTRATION OF THORIUM NITRATE TO MICE[1]

By S. J. Patrick[2]

With the Technical Assistance of Emma Marie Cross

Abstract

A method for the determination of microgram amounts of thorium in tissues by the use of the thorium isotope UX_1 was developed.

When an aqueous solution of thorium nitrate was administered orally to mice, the LD_{50} was found to be between 1.76 and 2.0 gm. of thorium per kgm. body weight. Mice supplied with a 0.1% solution of thorium nitrate as their only source of water exhibited a greater mortality than was found in a control group. The subcutaneous injection of 50 mgm. of thorium as a thorium nitrate solution into mice produced an acute, necrotizing, inflammatory reaction at the site of injection, but no other effects. The exposure of a group of mice to an aerosol of thorium nitrate solution on each of 90 days for 40 min. each day had no effect on their mortality. The average initial concentration of thorium for each exposure to the aerosol of thorium nitrate was 330 mgm. thorium per cu. m. of air and the average final concentration was 114 mgm. of thorium per cu. m. of air.

Evidence was obtained that all the thorium administered orally as thorium nitrate solution to mice is excreted by way of the feces and that no absorption from the gastrointestinal tract takes place. It is suggested that thorium nitrate owes its oral toxicity to its local action on the mucosa of the gastrointestinal tract.

No evidence was obtained that thorium was transported to the viscera and very little thorium was excreted after the subcutaneous injection of 0.5 mgm. of thorium as thorium nitrate solution into mice. Most of the thorium remained at the site of injection for at least seven days. The cutaneous application of 0.3 ml. of BAL (2,3-dimercaptopropanol) appeared to cause a slight increase in the excretion of thorium, but the distribution of thorium in the mouse was not affected by this treatment.

A group of mice was exposed for 180 min. to an aerosol of thorium nitrate that gave a maximum concentration of 107 mgm. of thorium per cu. m. of air and a final concentration of 3 mgm. of thorium per cu. m. of air. After this treatment the average amount of thorium found in and on one mouse was 256 μgm. Most of this was on the skin and most was excreted by way of the feces within a few days. Less than 0.2 μgm. of thorium was found in the liver, kidneys, spleen, blood, or femurs, immediately after the exposure or one day later. The average amount of thorium found in the lungs of one mouse immediately after the exposure was 3.12 μgm. and this amount decreased slowly over a period of 35 days.

Introduction

While a great deal of work has been done on the effects of colloidal thorium dioxide on men and animals, the literature contains few references to the metabolism of soluble thorium salts. The following investigation of the toxicity of thorium nitrate and the distribution and excretion of thorium after the administration of thorium nitrate to mice was therefore undertaken.

The Determination of Microgram Amounts of Thorium

An examination of the literature revealed few methods suitable for the determination of microgram amounts of thorium in tissues. Kuznetsov (2)

prepared the dye 2-hydroxy-3,6-disulphonaphthaleneazobenzene-2-arsonic acid, which gives a red color in the presence of thorium under acid conditions. A colorimetric method for the determination of 5 to 100 μgm. of thorium in 5 ml. of water was developed by Thomason, Perry, and Byerly (3) using this dye. In the present investigation the synthesis of the dye was accomplished through the following steps. o-Nitrophenylarsonic acid was prepared from o-nitroaniline and o-arsanilic acid was prepared from the o-nitrophenylarsonic acid by the method of Jacobs, Heidelberger, and Rolf (1). The dye was prepared by coupling the o-arsanilic acid with commercial "R" acid (2-naphthol-3,6-disulphonic acid) in the following manner. Two grams of arsanilic acid were dissolved in 5.0 ml. of 5 N hydrochloric acid and 1.0 gm. of sodium nitrite was dissolved in 4.0 ml. of water. Both solutions were cooled to 0° C. and were then combined. The resulting solution was neutralized by the dropwise addition of concentrated sodium hydroxide solution. A solution of 2.8 gm. of "R" acid in 20 ml. of 0.5% sodium hydroxide was added. The resultant dye was precipitated with two volumes of acetone, filtered, and dried *in vacuo*.

The determination of thorium by means of the dye was carried out by adding 1.0 ml. of a 0.1% solution of the dye in water to 0.2 ml. of concentrated hydrochloric acid in a 10 ml. volumetric flask, adding the solution containing the thorium and making up to volume with water. The color of the solution was compared with the color of a blank solution containing no thorium by means of a Klett photoelectric colorimeter using the 54 green filter. The amount of thorium in the solution was then determined by reference to a standard curve showing the relation between the colorimeter reading and amount of thorium in solution. It was found that the use of 1.0 ml. of the 0.1% dye solution and 0.2 ml. of concentrated hydrochloric acid permitted the convenient determination of 5 to 80 μgm. of thorium in 10 ml. of solution. Smaller amounts of dye decreased the range of the determination and strongly acid conditions resulted in a diminution of the color change. It was found that milligram amounts of sodium nitrate or nitrite, calcium nitrate, magnesium nitrate, barium nitrate, ammonium chloride, or potassium chloride did not interfere with the development of the color due to the reaction of 40 μgm. of thorium with 1.0 ml. of the dye solution. However, a few milligrams of sodium sulphate or phosphate decreased the color change.

Difficulty was encountered in the determination of thorium in tissue digests with this method. Substances present in the digests prevented the development of the color of the thorium complex. Therefore, this method was used for the determination of thorium only when no other materials were present in solution.

Other methods based on the precipitation of thorium with oxalic acid, "oxine", or other reagents are suitable only for larger quantities of thorium than were expected to be present in the material under investigation.

Thorium can be estimated by means of its radioactivity, but it was found that not less than 1 mgm. of thorium could be determined easily by counting the β particles with a Geiger-Mueller counter. However, the thorium isotope UX_1 can be easily prepared from uranium salts. This is an active β emitter, having a half-life of 24.6 days, and so can be used to trace very small amounts of thorium. In the following experiments the thorium compounds administered to the mice were enriched with the corresponding UX_1 compound and it was found a simple matter to determine the distribution of the thorium in the animal by determining the β activity of each tissue.

Table I shows the first part of the uranium series of radioactive changes and the relationship of UX_1 to its parent and daughter isotopes.

TABLE I

THE TRANSFORMATION OF URANIUM 238 BY RADIOACTIVE DECAY

Substance	Isotope	At. wt.	At. no.	Half-life	Type of disintegration	Absorption coeff. in Al
Uranium ↓	U	238	92	4.7×10^9y.	α	—
UX_1 ↓	Th	234	90	24.6 d.	β	463 cm.
UX_2 ↓	Pa	234	91	1.15 m.	β	14.4 cm.
U II ↓	U	234	92	2×10^6 y.	α	—
Io	Th	230	90	6.9×10^4y.	α	—

Since U II gives rise to a series of radioactive elements, none of which are uranium isotopes, and since U II and Io have extremely long half-lives, only the substances shown in Table I are present in measurable quantities in any freshly prepared uranium salt. Since UX_2 has such a short half-life, any sample of UX_1 is in equilibrium with a definite amount of UX_2 within a very short time after its isolation, but no other isotopes that emit β particles are present. A Geiger-Mueller counter of the "bell" type with an aluminum window 0.008 cm. thick was used to count the β particles. Under these conditions no α particles incident on the window can pass through, only a very small proportion of the β particles of UX_1 and about 89% of the β particles of UX_2 can pass through. Thus, the amount of UX_1 in any sample of material was determined by counting only the β particles emitted from the UX_2.

It was desirable to exclude the β particles of UX_1 from the counting because these are of low energy and so would be partially absorbed by substances in the tissue digests. The percentage absorption of the β particles of UX_1 would depend on the amount of digest present and thus the measured radioactivity of any tissue digest would depend not only on the amount of UX_1 present,

but also on the amount of inert material. The β particles of UX_2 are of high energy and it was shown that they were not appreciably absorbed by the amounts of tissue digests encountered in the experiments that were carried out.

UX_1 was separated from uranyl nitrate in the following manner. One hundred grams to 1 kgm. of uranyl nitrate hexahydrate was dissolved in peroxide free ether in a separatory funnel using 100 ml. of ether for every 100 gm. of uranyl nitrate hexahydrate. Two phases separated owing to the water of crystallization of the uranyl nitrate. The aqueous phase was found by β activity determination to contain practically all of the UX_1 and the ethereal phase contained most of the uranium. The aqueous phase was separated and made up in volume until it contained 5 gm. of uranyl nitrate per 100 ml. Ten mgm. of thorium as thorium nitrate was dissolved in the solution to serve as carrier for the UX_1. The solution was made 5 N with respect to hydrofluoric acid by addition of the concentrated acid and the solution was allowed to stand overnight. The precipitate of thorium fluoride was separated by centrifuging. It was found that most of the UX_1 was present in the precipitate. The precipitate was dissolved by heating with concentrated nitric acid and the solution was evaporated to dryness on the hot plate. The residue was dissolved in water, the thorium was reprecipitated with hydrofluoric acid, the precipitate was redissolved in nitric acid, and the nitric acid was boiled off. Thorium nitrate was added to the residue in sufficient quantity to give the desired specific radioactivity to the thorium. A solution of this material in water was used for the experiments on the distribution and excretion of thorium in mice.

The following method was used to determine the amount of enriched thorium in the tissues of the experimental animals. About 0.5 gm. of tissue was excised, weighed, and digested in a 30 ml. beaker with nitric and perchloric acid. The digestion was continued until the solution was practically colorless. The digest was boiled down until it was about 1 ml. in volume, and was transferred to a small Pyrex dish of internal diameter 2.5 cm. and with a wall 0.8 cm. high. The digest was evaporated to dryness with an infrared lamp. The beaker was washed with five successive portions of nitric acid, each of which was evaporated to dryness in the dish. The residue was thereby deposited in a suitably even layer over the bottom of the dish, and the weight of the residue was determined.

Dishes containing standard amounts of UX_1 were prepared and the amount of UX_1 in the tissue digest was determined by comparing the radioactivity of the digest in the dish with the radioactivity of the standard. The amount of thorium in the tissue digest was then calculated from the ratio of UX_1 to thorium in the material administered to the experimental animal.

Thorium in aqueous solutions or in urine was determined merely by pipetting an aliquot into a dish, evaporating the material to dryness, and determining its radioactivity.

Determinations of radioactivity were carried out in the following order: (1) a determination of the background counting rate, (2) a determination of the radioactivity of the standard sample of UX_1, (3) determinations of the radioactivity of the samples, (4) a redetermination of the radioactivity of the standard sample of UX_1. After use, each dish was cleaned by scrubbing with cleanser and water. The empty dish was then tested for radioactivity to make sure that no UX_1 was adhering to it.

Table II is a summary of experiments that were carried out in order to determine whether the amount or kind of inactive material in the dish had any effect on the measurement of UX_1. In these experiments, known amounts

TABLE II

THE DETERMINATION OF UX_1 IN THE PRESENCE OF MOUSE TISSUES

Tissue	No. of determinations	Amt. of tissue	Th in sample, μgm.	Method of determination	Average wt. of ash, mgm.	Average % recovery
Urine	4	0.5 ml.	20	Evaporation to dryness on dish	28	100
Urine	4	1.0 ml.	20	"	63	99
Urine	2	1.5 ml.	20	"	80	96
Urine	2	2.0 ml.	20	"	140	95
Blood	2	0.13 ml.	20	"	27	98
Blood	2	0.25 ml.	20	"	48	95
Blood	2	0.50 ml.	20	"	95	92
Liver	2	0.40 gm.	20	HNO_3, $HClO_4$ digestion	24	99
Liver	2	0.60 gm.	20	"	46	99
Intestine	2	0.40 gm.	20	"	27	99
Intestine	2	0.60 gm.	20	"	38	98
Kidney	2	0.40 gm.	40	"	25	97
Muscle	2	0.50 gm.	40	"	32	101
Skin	2	0.50 gm.	40	"	28	98
Bone (femurs)	2	0.10 gm.	40	"	87	99
Feces	2	0.10 gm.	20	"	55	100
Feces	2	0.20 gm.	20	"	120	94

of thorium enriched with known amounts of UX_1 were added to portions of mouse tissues, the UX_1 was determined, and the recovery of UX_1 was calculated. In subsequent experiments an amount of tissue was used such that the amount of residue in the dish was always less than 50 mgm. The data of Table II show that almost quantitative recoveries of UX_1 in the presence of tissues are obtained under these conditions.

The experiments shown in Table II were each carried out with an amount of UX_1 that gave about 1000 counts per minute under the conditions of counting that were used. The background counting rate was about 30 counts per minute and each sample was counted for 10 min. It is calculated that the odds are 20 : 1 that the error due to the random nature of the emission of β particles is less than $\pm 2\%$. Sometimes errors greater than this were

encountered in the determination of duplicate samples. These are attributed to factors such as unevenness in the disposition of the UX_1 and inert material in the dish and variation in counting efficiency. The sensitivity and accuracy of the determination of thorium was increased, when necessary, by increasing the ratio of UX_1 to thorium 232 in the material used.

The Toxicity of Thorium Nitrate for Mice

The acute toxicity of thorium nitrate after oral administration to mice was determined in the following manner. Groups of female albino mice were selected in such a manner that the greatest deviation of the weight of any mouse from the mean of the weight of all the mice in the group was one gram. Solutions of thorium nitrate in water containing approximately 100 mgm. of thorium per ml. were prepared. The exact concentration of thorium of each solution was determined by precipitating thorium oxalate from an aliquot part of the solution, igniting the thorium oxalate, and weighing the thorium oxide. A definite volume of the solution was administered to each mouse by means of a stomach tube consisting of a glass capillary tube, with an external diameter of about 1 mm., that was attached to a syringe by means of a short rubber tube. The mice were observed for two weeks following dosing. Pathological examinations were carried out on some of the mice that died. The results of these experiments are summarized in Table III.

TABLE III

THE TOXICITY OF THORIUM NITRATE AFTER ORAL ADMINISTRATION TO MICE

Av. wt. of mice in group, gm.	Conc. of solution, mgm. Th/ml.	Vol. of solution administered, ml.	Dose, gm. Th/kgm. body wt.	No. in group	No. dead in 14 days	Mortality, %
25	100.0	0.19	0.76	20	0	0
23	100.0	0.23	1.00	10	1	20
25	100.0	0.25	1.00	10	3	
20	100.0	0.25	1.25	20	6	30
29	101.1	0.43	1.50	10	1	35
29	98.8	0.44	1.50	10	6	
21	100.0	0.37	1.76	20	5	25
23	100.0	0.46	2.00	10	3	55
24	100.0	0.48	2.00	10	8	
25	100.7	0.56	2.25	20	18	90
23	100.7	0.57	2.50	20	20	100

A 0.8 ml. dose of a 10% solution of sodium chloride was administered to each of a group of 10 mice in order to discover any effects that might result from the administration of a comparatively large volume of a hypertonic solution. No effects were observed. Similarly no effects were observed after the administration of 0.8 ml. of a 10% solution of sodium nitrate to mice. It is concluded that the LD_{50} of thorium nitrate after oral administration to

mice is between 1.76 and 2.0 gm. of thorium per kgm. body weight and that the toxicity is due to the thorium ion.   Post-mortem examination showed occasional intestinal haemorrhage, but no damage to other internal organs.

An experiment was carried out to discover any effects of the continued ingestion of thorium nitrate.   In this experiment a group of 20 mice weighing between 24 and 26 gm. were supplied with 0.1% thorium nitrate solution as their only source of water.   It was found that this group of mice drank a volume of thorium nitrate solution comparable to the volume of water drunk by a control group of mice in the same length of time.   After a month no changes were noted in the animals receiving the thorium nitrate.   However, after four months 50% of the mice receiving the thorium nitrate were dead, whereas only 10% of the control mice were dead.   Although no lesions that could be ascribed to the action of thorium nitrate specifically were noted in the animals that died, this experiment suggests that continued ingestion of thorium nitrate has a deleterious effect.

The subcutaneous injection of 50 mgm. of thorium as thorium nitrate in 0.5 ml. of water into mice produced an acute, necrotizing, inflammatory reaction at the site of injection, but no lesions were noted in the viscera following this treatment.   None of the five mice treated in this manner died within 14 days after treatment.

An experiment was carried out to discover any effects of repeated exposures of mice to an aerosol of a thorium nitrate solution.   A group of 20 mice were exposed to the thorium nitrate aerosol on each of 90 week days for 40 min. each day.   Each exposure was carried out in the following manner.   Three ml. of a 10% solution of thorium nitrate in water were sprayed into a chamber of 125 liter capacity by means of a small glass atomizer using an air pressure of 18 lb. per sq. in.   The mice were then placed in the chamber in a wire cage with an individual compartment for each mouse.   The concentration of thor um in the air of the chamber was determined by passing 2.0 liters of the air through a gas washing bottle containing 25 ml. of water and determining the thorium content of the water.   The thorium content of the air in the chamber was determined again just before the mice were removed from the chamber 40 min. after they were put in.   The average initial concentration of thorium in the air in the chamber was 330 mgm. of thorium per cu. m. of air, and the average final concentration was 114 mgm. of thorium per cu. m. The mortality of these mice was not significantly different to that of a control group of mice.

The Distribution and Excretion of Thorium After the Administration of Thorium Nitrate to Mice

The following general methods were used in the experiments on the distribution and excretion of thorium after the administration of thorium nitrate to mice.   Female albino mice were used in all the experiments.   Aqueous solutions of thorium nitrate enriched with UX_1 nitrate were prepared and

the thorium content and radioactivity of each solution were determined by the methods that have been described. The activity of the UX_1 was such that one mouse was never exposed to more than about one μc.

After administration of the thorium nitrate either orally, subcutaneously, or by inhalation, a group of the treated mice were placed in a metabolism cage from which urine and feces could be collected and which was supplied with a water bottle. The mice were fed twice a day for one hour intervals in a separate cage. The urine and feces excreted during feeding periods were recovered from the bottom of the feeding cage. The feces were collected each day and dissolved by heating with nitric acid. Aliquot parts of this solution were analyzed for thorium. The urine was collected each day, centrifuged, and the supernatant urine and residue were analyzed separately.

Groups of mice were sacrificed at various intervals after the administration of the thorium nitrate. At this time each mouse was anesthetized, 0.05 ml. of heparin solution was injected into the jugular vein, and as much blood as possible was withdrawn from the vein by means of a 2.0 ml. syringe to which was attached a No. 23 needle. In most cases, the liver, kidneys, spleen, gastrointestinal tract and contents, lungs, and femurs were excised, freed of extraneous tissue, and placed in weighing bottles. The remainder of the carcass was placed in a weighed beaker. The tissues were cut into small pieces with scissors and weighed. Determinations of thorium were carried out on two aliquot portions of each tissue. The femurs were dissolved in hot nitric acid and thorium determinations were carried out on aliquot portions of the solution. The carcasses were treated in a similar manner.

Table IV is a summary of the results obtained in an experiment in which the excretion and distribution of thorium were determined for five mice, each of which was given orally 0.25 ml. of a solution of thorium nitrate containing 10 mgm. of thorium per ml. The distribution of thorium was determined seven days after dosing.

Table V is a summary of the results obtained in an experiment in which 0.25 ml. of a thorium nitrate solution containing 100 mgm. of thorium per ml. was administered orally to each of 10 mice. Five of the mice were sacrificed 24 hr. after dosing and five were sacrificed seven days after dosing.

The data obtained in these experiments suggest that all the thorium administered orally to mice as thorium nitrate is excreted by way of the feces with no absorption from the gastrointestinal tract. If this is the case the question arises as to why the administration of even large doses of thorium nitrate to mice results in their death. The following experiment threw some light on this question. Five mice were each given orally 0.25 ml. of a solution of thorium nitrate containing 100 mgm. of thorium per ml. The mice were sacrificed 24 hr. later and the gastrointestinal tracts were excised, opened, and cut in small pieces. This material was placed in a closed cheesecloth bag through which water was allowed to run for two hours. At this time, radioactivity measurements of the wash water showed that no more thorium was

being removed. The thorium content of the gastrointestinal tracts was then determined and was found to be 1.0% of the thorium administered to the mice. It is probable that this thorium had reacted with the proteins of the

TABLE IV

THE EXCRETION AND DISTRIBUTION OF THORIUM IN MICE
SEVEN DAYS AFTER THE ADMINISTRATION OF 100
MGM. OF THORIUM (AS THORIUM NITRATE)
PER KGM. BODY WEIGHT

Material analyzed	Administered thorium, %
Feces, Day 1	38.0
" " 2	34.0
" " 3	22.0
" " 4	1.2
" " 5	0.0
" " 6	0.0
" " 7	0.0
Total feces	95.2
Total urine	< 0.3
Total urine residue	0.39
Gastrointestinal tract	< 0.03
Liver	"
Kidneys	"
Spleen	"
Lungs	"
Femurs	"
Blood	"
Total in whole animal	< 0.2
Total recovery	95.59

mucosa. It is suggested that thorium nitrate owes its oral toxicity to its local action on the mucosa of the gastrointestinal tract. In the previous experiments, any thorium that reacted with the constituents of the mucosa was excreted in a few days. This may have been a result of sloughing off and excretion of the mucosa that was damaged by the thorium.

An experiment was carried out to determine the distribution and excretion of thorium in mice after the subcutaneous injection of thorium nitrate, and to discover whether the application of BAL (2,3-dimercaptopropanol) had any effect on this. In this experiment the hair of the back and flanks of each of a group of 16 mice was removed by clippers. A 0.5 ml. syringe to which was attached a No. 23 needle was filled with a thorium nitrate solution containing 2.0 mgm. of thorium per ml. The needle of the syringe was inserted under the skin of the right flank of a mouse and 0.25 ml. of the solution was injected. A clamp was then applied to the skin over the needle in such a manner as to prevent the solution from flowing back. The needle was withdrawn and a

small amount of "Glyptal" cement was applied over the hole in the skin left by the needle. When the cement was dry the clamp was removed. This technique prevented any of the solution from flowing to the surface of the

TABLE V

THE EXCRETION AND DISTRIBUTION OF THORIUM IN MICE AFTER THE ORAL
ADMINISTRATION OF 1000 MGM. OF THORIUM (AS THORIUM
NITRATE) PER KGM. BODY WEIGHT

Material analyzed	Administered thorium, %	
	In Group I sacrificed 24 hr. after dosing	In Group II sacrificed 7 days after dosing
Feces, Day 1	34.7	50.5
" " 2	—	36.5
" " 3	—	8.1
" " 4	—	0.13
" " 5	—	0.08
" " 6	—	0.07
" " 7	—	0.05
Total feces	34.7	95.43
Total urine	< 0.01	< 0.03
Total urine residue	0.06	0.80
Gastrointestinal tract and contents	56.3	< 0.03
Liver	< 0.03	< 0.03
Kidneys	"	"
Spleen	"	"
Lungs	"	"
Femurs	"	"
Blood	"	"
In remainder of carcass	< 0.2	< 0.2
Total recovery	91.06	96.23

skin. The treatment with BAL was carried out immediately after the injection of the thorium nitrate. A dose of 0.3 ml. of BAL, which had a sulphydryl content of 92% of theoretical by iodine titration, was applied to the skin of the left side of the back of the mouse over an area of about 6 sq. cm. The BAL was rubbed in for about three minutes with a glass rod. Sixteen mice were treated with thorium nitrate and eight of these were treated with BAL. Four of the mice that were treated with BAL and four of the mice that were not so treated were sacrificed after 24 hr. and the other eight mice were sacrificed after seven days. Thorium determinations were carried out on the excreta and the internal organs of each group of mice. The amount of thorium in the skin of each mouse at the site of injection of the thorium nitrate and the amount of thorium in each carcass was determined separately. The results of this experiment are summarized in Tables VI and VII. Mean values are quoted with the standard error of the mean.

The data of Tables VI and VII show no evidence that thorium was transported to the viscera after the subcutaneous injection of thorium nitrate and very little of the thorium was excreted. Most of the thorium appears to be

TABLE VI

THE DISTRIBUTION OF THORIUM AFTER THE SUBCUTANEOUS INJECTION OF THORIUM
NITRATE SOLUTION IN MICE TREATED WITH BAL AND IN UNTREATED MICE

Values are expressed as percentages of the administered thorium (0.5 mgm. per mouse)

Tissue	Time after dosing: 24 hr.		Time after dosing: 7 days	
	Mice treated with BAL	Untreated mice	Mice treated with BAL	Untreated mice
Skin at the site of injection	74 ± 6	77 ± 5	74 ± 6	80 ± 6
Carcass	21 ± 6	20 ± 6	15 ± 6	15 ± 5
Kidney	<0.05	<0.05	<0.05	<0.05
Liver	"	"	"	"
Spleen	"	"	"	"
Lungs	"	"	"	"
Gastrointestinal tract	"	"	"	"
Blood	"	"	"	"
Femurs	"	"	"	"

TABLE VII

THE EXCRETION OF THORIUM AFTER THE SUBCUTANEOUS INJECTION OF THORIUM
NITRATE SOLUTION IN MICE TREATED WITH BAL AND IN UNTREATED MICE

Values are expressed as percentages of the administered thorium (0.5 mgm. per mouse)

Day after dosing	Mice treated with BAL			Untreated mice		
	Urine	Urine residue	Feces	Urine	Urine residue	Feces
1	<0.1	0.03	0.18	<0.1	0.02	<0.1
2	<0.1	0.07	0.53	<0.1	<0.01	<0.1
3	<0.1	0.01	0.22	<0.1	0.01	<0.1
4	<0.1	0.01	<0.1	<0.1	<0.01	<0.1

fixed at the site of injection. The thorium found in the carcass was probably fixed to the subcutaneous tissue beneath the site of injection. The application of BAL did not significantly alter the distribution of thorium in the mice, but did appear to cause a small increase in the excretion of thorium in the feces.

An experiment was carried out to determine the distribution and excretion of thorium in mice after a single exposure to an aerosol of thorium nitrate solution. Twenty female mice were placed in a wire cage with an individual compartment for each mouse and the cage was placed in a closed cubical

chamber of 125 liter capacity. Three ml. of a solution of thorium nitrate containing 10 mgm. of thorium per ml. and with a UX_1 activity of about 9.4 μc. per ml. was placed in each of three small glass atomizers. These atomizers were of the type with a glass baffle, and were obtained from the Vaponefrin Co. The thorium nitrate solution in each atomizer was sprayed into the chamber through a port near the top using an air pressure of 18 lb. per sq. in. An attempt was made to determine the approximate size of the droplets of solution by placing microscope slides in the chamber for short intervals and measuring the size of the droplets on the slides by means of a microscope with a calibrated scale in the eyepiece. It was concluded that the diameter of almost all the droplets was less than 3 μ, the majority being less than 1 μ.

All the thorium nitrate solution was sprayed into the chamber 35 min. after the start of the spraying and the mice were removed from the chamber 180 min. after the start of the spraying. Table VIII shows the concentration

TABLE VIII

THE CONCENTRATION OF THORIUM IN THE AIR OF THE
CHAMBER IN WHICH MICE WERE EXPOSED TO AN
AEROSOL OF THORIUM NITRATE SOLUTION

Time after the start of the spraying, min.	Concentration of thorium, mgm. of thorium/cu. m. of air
15	82
35	107
50	64
65	42
80	30
95	19
110	15
180	3

of thorium in the air of the chamber at various intervals after the start of the spraying. Each of these determinations was carried out by withdrawing a 2 liter sample of air from a port near the bottom of the chamber. The sample of air was passed at the rate of 0.5 liter per minute through a gas washing bottle containing 25 ml. of water and the thorium content of the water was determined. Previous experiments had shown that all the thorium in the sample of air was absorbed by the water in the absorber under these conditions. Since the port through which the air samples were withdrawn was at the same level as the mice, it is probable that the values quoted in Table VIII are accurate estimations of the concentration of thorium in the air that the mice were breathing.

Groups of four mice each were sacrificed at the following times after the exposure: 5 min., 24 hr., 7 days, 21 days, and 35 days. Each mouse in the group was treated in the following manner at the time of sacrifice. The

blood was withdrawn and the skin was removed as completely as possible. The tail and paws to which some skin adhered were added to the skin. The head without any skin, the lungs, gastrointestinal tract, liver, kidneys, spleen, and femurs were excised. Thorium determinations were carried out on the organs and the remainder of the carcasses of the four mice in the group. Four of the mice were placed in a metabolism cage immediately after the exposure and urine and feces were collected for seven days after the exposure. The results of these experiments are summarized in Tables IX and X.

TABLE IX

THE DISTRIBUTION OF THORIUM IN MICE AFTER EXPOSURE OF THE MICE
TO AN AEROSOL OF THORIUM NITRATE SOLUTION

Results expressed as μgm. of thorium in the tissues of one mouse

Tissue	Time after the start of exposure				
	3 hr.	24 hr.	7 days	21 days	35 days
Skin, tail, and paws	149	24.6	3.5	1.6	<0.7
Head without skin	14.8	1.1	<0.5	<0.5	<0.5
Lungs	3.10	2.60	2.25	1.83	1.38
Gastrointestinal tract and contents	85.6	.7.4	<0.5	<0.5	<0.5
Liver	<0.2	<0.2	<0.2	<0.2	<0.2
Kidneys	"	"	"	"	"
Spleen	"	"	"	"	"
Femurs	"	"	"	"	"
Blood	"	"	"	"	"
Remainder of carcass	4.7	<1.0	<1.0	<1.0	<1.0
Total	257	36	6	3	1

TABLE X

THE EXCRETION OF THORIUM AFTER THE EXPOSURE OF MICE TO AN
AEROSOL OF THORIUM NITRATE SOLUTION

Results expressed as μgm. of thorium excreted by one mouse

Day after the exposure	Urine, μgm. Th	Feces, μgm. Th
1	<0.2	152
2	<0.1	51
3	<0.2	16
4	<0.1	13
5	<0.1	1
6	<0.1	3
7	<0.1	2
Total	<0.9	238

The data in Tables IX and X show no evidence that thorium reaches the liver, kidney, spleen, bone, or blood after exposure of mice to an aerosol of thorium nitrate. It is probable that the small amount of thorium found in the carcasses of the mice five minutes after exposure owed its presence there to contamination from the fur or gastrointestinal tract and that no thorium was actually present in the skeletal muscle or bones of the intact mice. A comparatively large amount of thorium was deposited on the fur of the mice and it is probable that the mice transferred this thorium to the gastrointestinal tract by licking. This thorium was then excreted by way of the feces and very little or no absorption of the thorium took place. The thorium found in the head was probably in the upper parts of the respiratory and gastrointestinal tracts. Almost all of the thorium in and on the mouse was eliminated by way of the feces within a week after the exposure. A small amount of thorium was found in the lungs and this decreased slowly over a period of 35 days after the exposure. It is probable that this thorium reacted with the constituents of the lung tissue.

Acknowledgments

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"DROP PLATE" METHOD OF COUNTING VIABLE BACTERIA[1]

BY R. W. REED[2] AND G. B. REED[3]

Abstract

This paper is a critical review of the "drop plate" method of determining the number of viable bacteria in fluids, together with a description of an experimental comparison of the "drop plate" with the more usual "pour plate" procedure. It is shown that counts on pure cultures of bacteria made by the "drop plate" method are some 7% higher than those made on the same cultures by the "pour plate" method. It is also shown that the standard error of a series of counts made by the "drop plate" procedure is slightly less than for those made by the "pour plate" method.

A successful "drop plate" method of determining the number of viable bacteria in fluid suspension has been in use for a number of years in several English research laboratories. Considerable experience with the method suggests the desirability of reviewing the procedure both as a research tool and for routine assays of such material as milk.

Donald (2) introduced a method for the precise measurement of fluid volume by means of drops. This depends upon the fact that the size of a drop delivered from a pipette is governed, other factors being constant, by the *external* diameter of the dropping tube. Fildes and Smart (3) expanded the procedure and developed methods of preparing and calibrating the pipette. Several authors have described in brief form the adaptation of dropping pipettes to the technique of plate counts of bacteria, particularly Wilson (9), Aitken, Barling, and Miles (1), Kenny, Johnston, von Haebler, and Miles (5), von Haebler and Miles (4), Miles, Misra, and Irwin (7), and Snyder (8). The last two papers include a statistical analysis of the accuracy of the method.

The plating procedure consists simply in adding, with a calibrated dropping pipette, drops of properly diluted suspensions of bacteria to the surface of nutrient agar or other appropriate medium in Petri plates. The surface of the medium must be dried to the stage where a drop of bacterial suspension will spread over an area of 1.5 to 3 cm. in diameter and permit absorption of the fluid of the drop in 15 to 20 min. On incubation the area covered by a drop of suitably diluted suspension will develop well spaced surface colonies that permit rapid and accurate counts.

Methods

Dropping Pipettes

These are made, as described by Donald, by drawing a length of 7 mm. glass tubing to form two pipettes with long, very gradual tapers. They may

be sized with a wire gauge; however, we find more convenient a square of steel ⅛ in. thick with one hole 0.047 in. in diameter. The tapered portion of the pipette is pushed through the gauge hole until it fits snugly, scratched with a sharp file or glass knife at the margin of the gauge, removed, and broken. The external diameter of the pipette should then be very close to that of the gauge, provided the glass is drawn with a very gradual taper and is circular in cross section. The tip must be uniform and at right angles to the axis of the pipette. With practice these may be made rapidly and accurately.

The pipettes are manipulated with a rubber teat and must be held in a perpendicular position when drops are delivered. They may be calibrated by dropping a counted number of drops into a tared weighing bottle. In this procedure, as indicated by Fildes and Smart (3), it is essential to deliver not more than 30 drops a minute. Pipettes made with the 0.047 in. gauge deliver 40 drops of water per ml.

A set of pipettes may be used repeatedly provided they are scrupulously cleaned. Our practice both with the dropping pipettes and with graduated diluting pipettes is to discard used pipettes into Roccal solution, wash them in running water in an automatic washer, allow them to stand for one to two hours in chromate cleaning mixture, and wash them overnight in running water. They are then dried, loosely plugged with cotton, and sterilized in the ordinary manner.

Plating Medium

Several types of media were used in the experiments reported in this paper. In order to prepare different lots as uniform as possible each type was made from accurately weighed amounts of Difco dehydrated medium bearing the same 'batch' numbers.

Drying Plates

The time and temperature of drying the plates must vary with the relative humidity of the atmosphere. In the greater part of this work, with a relative humidity of 60 to 75%, optimum surfaces were obtained by drying recently poured and cooled plates, 20 ml. agar per plate, for one hour at 37° C. In a few cases where the relative humidity ranged from 75 to 100%, satisfactory results were obtained by drying for one hour at 75° C. In all work reported in this paper, plates were dried, tilted upside down on their lids in the drying chamber.

This drying time was arrived at from the results of the experiment summarized in Table I, on counts on a fluid culture of *Shigella alkalescens*. Plates were poured, 15 ml. and 25 ml. per plate and dried, as just described, at 37° for 30, 60, and 90 min. It is apparent from the table that drying within this range has no significant influence on the counts.

TABLE I

EFFECT OF AMOUNT OF AGAR AND DRYING TIME OF PLATES ON DROP COUNT OF *Shigella alkalescens*

		Counts, $\times 10^8$			
15 ml. of agar per plate			25 ml. of agar per plate		
Drying time at 37° C., min.					
30	60	90	30	60	90
9.12	9.06	9.50	9.54	8.66	8.86
8.36	8.40	9.74	8.96	8.50	9.10
8.70	8.50	8.76	9.06	9.16	8.80
Mean 8.73	8.65	9.33	9.19	8.77	8.92
Standard error 0.18	0.17	0.23	0.15	0.16	0.07

Dilutions

The number of colonies developing per drop of fluid added to the plates that can be most satisfactorily counted is 20 to 40. The aim, therefore, in making dilutions is to cover the range of 20 to 40 organisms per drop, i.e. per 1/40th ml. Ordinarily serial 1–10 dilutions were made, or for the most accurate work, especially where the approximate range of concentration of bacteria was known, serial 1–2 dilutions were made. The number of these dilutions used for plating depends on how accurate an estimate can be made of the concentration of bacteria present.

Dilutions were made by adding 1 ml. to a 9 ml. blank in a 6 $\times$ ¾ in. test tube and mixing, with the pipette, used for the next transfer, by alternately filling and blowing out 10 times. Two fluids were tried for dilution blanks, distilled water and buffered gelatin–saline*. In a number of parallel counts from a single culture of *Shigella alkalescens* using the two dilution fluids the following total counts were obtained:

Diluting fluid	Mean count per ml. of culture
Distilled water	$4.10 \pm 0.18 \times 10^8$
Gelatin–saline	$4.47 \pm 0.20 \times 10^8$

In all subsequent counts recorded in this paper gelatin–saline was used for the dilutions.

* *Sodium chloride*	*0.8 gm.*
Sodium dipotassium phosphate	*0.2 gm.*
Gelatin	*0.1 gm.*
Distilled water	*100 ml.*

Plating

After the dilutions are made and plates of appropriate medium dried, drops of the selected dilutions are added to the plates with calibrated dropping pipettes. Usually six drops of one dilution are added to a plate of agar. In delivering measured drops the most satisfactory procedure is to hold the pipette in a perpendicular position with the tip about 5 mm. above the surface of the agar. It is essential that the pipette be held firmly in a fixed position so that the drops form their maximum size and fall freely from the tip of the pipette. The drops should be evenly spaced on the agar so that they neither coalesce nor extend to the margin of the plate. Within one to two minutes after the drops are delivered the plates should be rotated while firmly held to the bench surface, so as to spread the drop over an area of 1.5 to 3 cm. in diameter. The plates are allowed to stand on the bench in an upright position for 15 to 20 min. to permit absorption of the fluid by the agar. They are then inverted and incubated 16 to 20 hr. or longer in the case of slow growing species.

Counting

Counts are conveniently made with the aid of a colony counter in the ordinary manner. The total number of bacteria per ml. or per gm. of the original

$$\text{material} = \frac{A}{B} \times C \times D$$

Where A = number of colonies per plate
        B = number of drops per plate (usually 6)
        C = number of drops per ml. (usually 40)
        D = the dilution factor

Accuracy of Method

A number of experiments were carried out to test the accuracy of the drop method and to compare it with the usual pour plate method of counting.

Six operators having varying degrees of experience with the drop count procedure each prepared a series of dilutions to 1–400,000 in saline–gelatin blanks from the same 24-hr. broth culture of *Salmonella typhimurium*, then prepared six nutrient agar plates, six drops per plate, using three dropping pipettes. Results are shown in Table II. The mean values obtained by the six operators vary from 7.49 to 8.35 × 10^8 and the standard errors of each mean vary from 0.11 to 0.24 × 10^8. It is therefore evident that the difference between the mean values is the order of five times the standard error of each operator's counts.

Comparison of "Drop" and "Pour" Counts

A series of counts were made by five operators on cultures of *Shigella alkalescens, Shigella dysenterae*, and *Salmonella typhimurium*. In each instance the five operators worked from the same 24-hr. broth culture. Each operator

TABLE II

DROP PLATING OF S. *typhimurium* ON BACTO-NUTRIENT AGAR BY SIX OPERATORS OF VARYING
DEGREES OF EXPERIENCE. DILUTION 1 : 400,000

Operator	Counts, $\times$ 10^8					
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
	8.00	7.80	8.08	7.41	7.17	8.14
	7.48	7.57	7.68	8.04	7.64	9.39
	9.31	6.93	7.54	8.18	7.92	8.24
	8.14	8.12	8.24	8.04	7.20	7.80
	8.77	8.64	7.80	7.34	7.96	7.73
	8.28	7.48	—	7.84	7.06	8.82
Mean	8.33	7.76	7.97	7.81	7.49	8.35
Standard error	0.24	0.22	0.11	0.13	0.13	0.24

NOTE: *Mean count, all plates = 7.95 $\times$ 10^8.*
Standard error = 0.051 $\times$ 10^8.

made one set of dilutions and prepared "drop" and "pour" plates from this
one set of dilutions. The same lot of medium was used for the two types of
plates. Differences between the counts by the two methods must therefore
result from the actual plating and growth on the plates rather than from
diluting procedures. The results are summarized in Tables III to V.

TABLE III

COMPARISON OF "DROP" AND "POUR" PLATING OF *Shigella alkalescens* ON BACTO-NUTRIENT
AGAR BY FIVE OPERATORS. DROP PLATES MADE FROM 1 : 500,000 DILUTION, POUR
PLATES FROM 1 : 3,000,000

Operator	Counts, $\times$ 10^8									
	No. 1		No. 2		No. 3		No. 4		No. 5	
	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"
	5.34	5.19	5.62	6.03	5.60	5.22	6.00	4.05	5.14	5.76
	5.66	5.01	5.20	5.31	4.86	6.21	6.46	4.56	5.60	4.98
	4.74	4.50	5.36	5.94	5.40	5.52	5.56	4.77	5.14	4.77
	5.40	5.91	6.30	5.52	5.16	5.37	4.70	4.29	4.90	5.34
	5.36	5.04	5.96		5.54	5.58	4.42	4.02	5.60	5.55
	4.56	5.31	4.62		5.40	5.19	5.30		4.80	4.92
Mean	5.18	5.16	5.51	5.56	5.33	5.52	5.41	4.42	5.20	5.22
Standard error	0.16	0.17	0.22	0.16	0.09	0.14	0.29	0.12	0.13	0.15

	Mean	Standard error
30 "Drop" plates	5.33 $\times$ 10^8	0.022 $\times$ 10^8
27 "Pour" plates	5.18 $\times$ 10^8	0.078 $\times$ 10^8

TABLE IV

COMPARISON OF "DROP" AND "POUR" PLATING OF *Shigella dysenteriae* ON BACTO-NUTRIENT AGAR BY FIVE OPERATORS. DROP PLATES MADE FROM 1 : 500,000 DILUTION, POUR PLATES FROM 1 : 3,000,000

Operator	Counts, $\times 10^8$									
	No. 1		No. 2		No. 3		No. 4		No. 5	
	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"
	4.96	5.13	4.80	4.56	5.96	5.31	4.60	5.46	5.36	4.74
	5.00	5.40	5.96	4.35	5.90	4.92	5.06	5.10	5.40	5.67
	5.54	5.43	6.10	4.50	6.16	5.52	4.70	4.17	4.42	4.89
	5.34	4.95	5.26	4.77	5.66	5.43	4.22	4.29	5.20	4.71
	5.94	4.89	6.10	4.74	4.70	5.16	5.56	4.44	5.42	6.24
	5.54	5.01	5.10	5.13	5.34	4.89	5.16	4.56	5.06	5.13
Mean	5.39	5.15	5.57	4.68	5.62	5.21	4.88	4.67	5.14	5.23
Standard error	0.14	0.09	0.21	0.11	0.19	0.10	0.17	0.19	0.14	0.22

	Mean	Standard error
30 "Drop" plates	5.32×10^8	0.051×10^8
30 "Pour" plates	4.95×10^8	0.048×10^8

TABLE V

COMPARISON OF "DROP" AND "POUR" PLATING OF *Salmonella typhimurium* ON BACTO-NUTRIENT AGAR BY SIX OPERATORS. DROP PLATES MADE FROM 1 : 500,000 DILUTION AND POUR PLATES FROM 1 : 3,000,000

Operator	Counts, $\times 10^8$											
	No. 1		No. 2		No. 3		No. 4		No. 5		No. 6	
	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"	"Drop"	"Pour"
	7.14	6.72	6.74	6.00	6.54	5.52	6.40	6.66	7.82	7.20	7.62	7.68
	7.36	7.23	7.22	6.84	6.54	4.83	6.54	6.33	6.94	6.60	7.46	7.05
		6.90	7.70	6.30	7.56	4.56	7.36	6.06	7.40	6.87	7.40	6.41
		7.59	7.02	6.27	7.26	6.12		5.52	7.56	7.71		6.84
		6.96	7.10	5.73		5.85		7.23	6.76	7.23		5.67
		7.53	7.34	5.67		5.46		6.18	7.26	6.84		7.11
			7.66									
Mean	7.25	7.12	7.25	6.14	7.47	5.39	6.77	6.33	7.29	7.08	7.49	6.79
Standard error	0.08	0.13	0.12	0.16	0.34	0.17	0.24	0.21	0.15	0.15	0.06	0.26

	Mean	Standard error
23 "Drop" plates	7.25×10^8	0.05×10^8
36 "Pour" plates	6.48×10^8	0.10×10^8

The five operators, Table III, obtained "drop" counts on one culture of *Shigella alkalescens* with mean values of 5.18 × 10⁸ to 5.51 × 10⁸ and with standard errors of 0.14 to 0.21 × 10⁸ per ml. of culture, and "pour" counts with mean values of 4.42 × 10⁸ to 5.56 × 10⁸ with standard errors of 0.09 to 0.22 × 10⁸. Similar relative values were obtained by the two methods of counting cultures of *Shigella dysenterae*, Table IV, and *Salmonella typhimurium*, Table V. In the summaries at the bottom of Tables III to V it will be noted that in each instance the mean of the "drop" count is 3 to 10% higher than the mean of the "pour" count. It will also be noted from these summaries that in each instance the standard error of the mean of the "drop" counts is slightly less than the standard error of the mean of the "pour" counts.

Counts made on two lots of *Bacillus subtilis* spores stored in semi-dry fine ground peat (Lochhead and Thexton (6)) for 15 and 24 months and for 12 and 19 months were made on Difco heart infusion agar and on the regular laboratory heart infusion agar. As in former determinations the counts shown in Table VII indicate mean values for the "drop" plate counts slightly higher than the means of the "pour" plate counts but counts on another lot of similar material, shown in Table VI, indicate the reverse, i.e., mean values of the

TABLE VI

COMPARISON OF "DROP" AND "POUR" PLATING OF *Bacillus subtilis* ON TWO TYPES OF AGAR AFTER 15 AND 24 MONTHS' STORAGE IN PEAT AT 28% MOISTURE

Counts, ×10⁹

	15 months storage in peat				24 months storage in peat			
	Drop plates		Pour plates		Drop plates		Pour plates	
	1 : 4,000,000 dil.		1 : 20,000,000 dil.		1 : 2,000,000 dil.		1 : 20,000,000 dil.	
	Difco infusion	Lab. infusion	Difco infusion	Lab. infusion	Difco infusion	Lab. infusion	Difco infusion	Lab. infusion
	2.96	3.39	3.28	3.06	2.49	3.11	4.56	3.50
	3.07	2.99	3.28	3.00	3.97	3.24	3.66	4.20
	3.31	3.76	3.16	2.94	3.40	3.43	4.20	3.78
	3.36	3.39		2.24	3.86	2.93	4.16	3.64
	3.07	3.07		3.12	3.13	2.77	3.78	3.74
					3.66	2.99	3.68	3.54
Mean	3.15	3.32	3.24	2.87	3.42	3.08	4.01	3.73
Standard error	0.07	0.12	0.03	0.15	0.20	0.08	0.13	0.09

"drop" plate counts are slightly lower than the mean values of the "pour" plate counts. This is the only case in many comparative counts where the "drop" plate counts have not been higher than the "pour" plate counts. It is apparent from the tables that the standard error of the two methods of counting *B. subtilis* is of the same order.

TABLE VII

COMPARISON OF "DROP" AND "POUR" PLATING OF *Bacillus subtilis* ON TWO TYPES OF AGAR
AFTER 12 AND 19 MONTHS' STORAGE IN PEAT AT 25% MOISTURE

	Counts, $\times 10^8$							
	12 months storage in peat				19 months storage in peat			
	Drop plates		Pour plates		Drop plates		Pour plates	
	1 : 600,000 dil.		1 : 5,000,000 dil.		1 : 300,000 dil.		1 : 3,000,000 dil.	
	Difco infusion	Lab. infusion	Difco infusion	Lab. infusion	Difco infusion	Lab. infusion	Difco infusion	Lab. infusion
	12.32	13.60	10.45	12.75	3.86	3.94	2.55	2.49
	13.48	13.60	11.30	11.35	3.06	3.28	4.23	3.09
	11.80	12.04	12.15	10.00	3.90	3.64	3.78	3.03
	11.72	11.56	11.50	10.90	3.46	3.56	3.60	2.97
	12.20	13.24	10.70	11.85	3.94	3.28	3.12	2.76
	11.08	13.28	9.75	9.85	3.90	3.70	3.48	2.76
					3.48	3.46	3.63	3.30
					3.64	3.22		
Mean	12.10	12.55	10.98	11.12	3.66	3.52	3.48	2.91
Standard error	0.30	0.35	0.31	0.41	0.09	0.08	0.18	0.09

Counts on Differential Media

Counts were made on cultures of *Shigella alkalescens*, *Shigella dysenterae*, and *Salmonella typhimurium* on blood agar, MacConkey's, Endo's, and Difco S.S. agar in contrast with Difco heart infusion and laboratory heart infusion agars primarily to determine the significance of counts on differential media.

It is apparent from the results shown in Tables VIII, IX, and X that consistent counts may be made by the "drop" method on opaque media. It is

TABLE VIII

COMPARISON OF "DROP" AND "POUR" PLATING OF *Shigella alkalescens* ON SEVERAL TYPES
OF BACTO AGAR. "DROP" PLATES FROM 1:500,000 DILUTION, "POUR" PLATES
FROM 1 : 3,000,000

	Counts on drop plates, $\times 10^8$					Counts on pour plates, $\times 10^8$	
	Difco infusion	Lab. infusion	Blood	Mac-Conkey	Endo	Difco infusion	Lab. infusion
	5.10	5.14	5.36	5.76	5.20	4.32	4.77
	5.70	5.60	5.44	4.56	5.60	5.55	5.76
	5.00	5.14	5.50	5.74	4.84	4.86	4.98
	5.02	4.90	5.66	5.20	4.50	5.31	5.34
	4.42	5.60	5.10	4.94	5.16	4.53	5.55
	5.62	4.80	5.96	5.66	4.60	4.47	4.92
Mean	5.14	5.20	5.50	5.31	4.98	4.84	5.22
Standard error	0.18	0.15	0.11	0.19	0.18	0.18	0.15

TABLE IX

COMPARISON OF "DROP" AND "POUR" PLATING OF *Shigella dysenteriae* ON SEVERAL TYPES OF AGAR. "DROP" PLATES MADE FROM 1 : 500,000 DILUTION, "POUR" PLATES FROM 1 : 3,000,000

	Counts on drop plates, $\times 10^8$					Counts on pour plates, $\times 10^8$	
	Difco infusion	Lab. infusion	Blood	Mac-Conkey	S S	Difco infusion	Lab. infusion
	5.56	5.36	6.50	4.96	2.14	6.54	4.74
	5.94	5.40	6.14	5.10	2.54	5.79	5.67
	5.96	4.44	6.50	5.16	1.46	5.19	4.89
	5.96	5.20	5.90	4.96	1.86	4.83	4.71
	5.76	5.44	6.36	3.90	2.04	5.67	6.24
	6.56	4.56	6.46	5.90	2.14	5.61	5.13
Mean	5.96	5.10	6.31	5.00	2.03	5.61	5.23
Standard error	0.12	0.17	0.09	0.23	0.13	0.22	0.23

TABLE X

COMPARISON OF "DROP" AND "POUR" PLATING OF *Salmonella typhimurium* ON SEVERAL TYPES OF AGAR. "DROP" PLATES MADE FROM 1 : 500,000 DILUTION, "POUR" PLATES FROM 1 : 3,000,000

	Counts on drop plates, $\times 10^8$						Counts on pour plates, $\times 10^8$	
	Difco infusion	Lab. infusion	Blood	Mac-Conkey	Endo	S S	Difco infusion	Lab. infusion
	7.06	7.64	7.14	7.70	7.64	4.14	8.01	7.68
	7.80	7.44	6.30	6.96	7.54	3.84	7.74	7.05
	6.86	7.44	7.84	6.04	7.10	4.70	7.26	6.67
	7.16		7.46	6.96	7.16	3.66	6.84	7.14
	7.10		6.96	7.20	8.00	3.74	7.14	5.67
	7.76		7.66	6.46	6.96	3.86	8.38	7.11
Mean	7.29	7.51	7.23	6.88	7.40	3.99	7.48	6.89
Standard error	0.15	0.05	0.26	0.21	0.15	0.14	0.23	0.25

also apparent that with these three species MacConkey's medium gives only slightly lower counts than the standard media but that Endo's and Difco S.S. media give by contrast counts that are too low to be significant.

Duplicate counts made by "drop" and "pour" methods on heart infusion and nutrient agars again show, Tables VIII, IX, and X, that the "drop" method gives slightly higher mean counts than the "pour" method. The tables also show that the standard error of "drop" counts is appreciably lower than the standard error of "pour" counts.

Counts of Other Groups of Bacteria

In addition to the four species mentioned in this paper successful "drop" counts have been carried out on one to several species belonging to the following groups of bacteria.

Group	*Medium*
Staphylococcus	Infusion agar
Streptococcus	Infusion agar
Pneumococcus	Blood agar
Serratia	Infusion agar
Pseudomonas	Infusion agar
Achromobacter	Infusion agar
Proteus	Infusion agar
Salmonella	Infusion agar
Eberthella	Infusion agar
Shigella	Infusion agar
Lactobacillus	Milk agar
Pasteurella	Tryptose agar.
Brucella	Tryptose agar
Bacillus	Infusion agar

The method appears to be applicable to any aerobic form. The method has been tried with *Clostridium*, with and without a reducing agent in the plating medium, with the plates incubated in Fildes type anaerobic jars. However, the counts were always much lower than with "pour" plates made from the same agar and incubated in the same anaerobic jar.

Conclusion

It is apparent from these results that the "drop" method of making counts of bacteria has some advantages over the conventional "pour" plate method: it is less laborious, it is a little more accurate, with most of the species tested the counts are appreciably higher, and it is applicable to opaque as well as transparent media.

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COMPARISON OF "DROP" AND "POUR" PLATE
COUNTS OF BACTERIA IN RAW MILK[1]

By J. J. R. Campbell[2] and J. Konowalchuk[3]

Abstract

In the preceding paper it was shown that a "drop plate" method of determining the number of viable bacteria in pure cultures gives slightly higher counts than the usual "pour plate" method. In this paper it is shown that, in parallel counts made by the two methods on a series of samples of raw milk, the "drop plate" counts are some 27% higher than the "pour plate" counts. It is suggested that this discrepancy results from the more efficient breaking up of clumps and chains of bacteria by the dilution procedure used in preparing the "drop plates."

In the preceding paper, Reed and Reed (1), the technique of "drop plate" assays for viable bacteria as applied to pure cultures was reviewed and the results compared with those obtained in the conventional "pour plate" method. It was demonstrated that the "drop plate" method is slightly more accurate than the "pour plate" method; that the labor involved in preparing the plates is less; and that colony counts are made with greater certainty in the "drop plate" than in the "pour plate" procedure. Since all these comparisons were made with pure cultures as the plating material it seemed desirable to compare the two methods on some natural product that exhibits a mixed flora. Raw milk was chosen and the following experiments were carried out.

Individual samples of raw milk from 28 different producers were collected as delivered at a local dairy. Duplicate "drop plate" counts were made on each sample by an operator with long experience with the method. In this procedure dilutions were made, as described by Reed and Reed (1) by adding 1 ml. to 9 ml. blanks and mixing by alternately filling and blowing out the pipette 10 times, the pipette used for the next transfer. Conventional "pour plate" counts were made in duplicate on the same samples of milk by an operator with long experience in making routine counts by this method. For the "pour plates" dilutions were made by adding 1 ml. to 99 ml. blanks in square dilution bottles and mixing by three minutes hand shaking. Dilution blanks and agar for both methods were from the same batches of material. Agar prepared according to Standard Methods of milk analysis was used for both sets of plates and incubation of plates was in accordance with Standard Methods. Not more than two to three minutes elapsed between the time a sample of milk was plated by one operator and the time it was plated by the other. The time required to prepare dilutions and the plates was approximately the same for the two methods. Colony counts were made somewhat more rapidly on the "drop plates."

Results summarized in the table indicate that the mean count for all samples plated by the "drop plate" method was 27% higher than that for the same samples plated by standard methods. The mean deviation per sample with duplicate counts is greater with the "drop plate" than with the "pour plate" method. This confirms and extends the conclusions of the previous paper that the "drop plate" method gives higher counts than the conventional method. It is also shown that with a mixed flora, such as occurs in raw milk, one can expect even greater differences in count between the two methods than with pure cultures of the organisms used by Reed and Reed (1).

Since a method that favors only bacteria that thrive in air gives a higher count than the standard plating procedure, even when the sample being assayed contains a predominance of lactic acid bacteria, which are inhibited by air, then there must be more serious limitations to the standard plate count than is now recognized. There are several possible explanations. One is that agar at 43° C. to 45° C. lowers the count more than is believed. Another possible explanation is that many aerobic types are not given an opportunity to grow on a pour plate. However, this should not be true with these samples of milk where the overwhelming majority of the organisms appear to be either lactic acid bacteria or members of the colon aerogenes group. The most reasonable explanation would seem to be that the pipetting technique used in mixing the dilutions in the "drop method" is much more efficient in breaking up chains and clumps of cells than is the usual shaking technique.

TABLE I

COMPARISON OF "DROP" AND "POUR" PLATING OF RAW MILK SAMPLES

Milk sample No.	"Drop plates"			"Pour plates"		
	Count/ml., × 1000	Mean, × 1000	Mean deviation, × 1000	Count/ml., × 1000	Mean, × 1000	Mean deviation, × 1000
1	4800 2800	3800	±1000	2300 2500	2400	100
2	4400 11,000	7700	±3300	2500 2450	2475	25
3	520 600	560	± 40	500 500	500	0
4	320 280	300	± 20	125 140	132.5	7.5
5	68 92	80	12	85 75	80	5
6	20 32	26	6	14.5 15.5	15	0.5
7	28 24	26	2	17.5 18	17.75	0.25
8	4 4	4	0	5.5 6.2	5.85	0.35

	Drop plates	Pour plates
Note: *Mean count per sample*	*1,482,280*	*1,077,600*
Mean deviation per sample	*264,140*	*119,000*

TABLE I—*Concluded*

COMPARISON OF "DROP" AND "POUR" PLATING OF RAW MILK SAMPLES—*Concluded*

Milk sample No.	"Drop plates"			"Pour plates"		
	Count/ml., × 1000	Mean, × 1000	Mean deviation, × 1000	Count/ml., × 1000	Mean, × 1000	Mean deviation, × 1000
9	640 440	540	100	1000 1050	1025	25
10	3200 3600	3400	200	4500 5000	4750	250
11	360 320	340	20	25 25	25	0
12	5600 5600	5600	0	3000 3500	3250	250
13	160 160	160	0	40 70	55	15
14	20 16	18	2	7.5 8.0	7.75	0.25
15	16 24	20	4	4.5 5.0	4.75	0.25
16	2800 3600	3200	400	4000 2900	3450	550
17	200 60	130	70	75 100	87.5	12.5
18	160 160	160	0	60 55	57.5	2.5
19	400 280	340	60	420 380	400	20
20	40 40	40	0	33 25	29	4
21	7600 6800	7200	400	1500 1450	1475	25
22	2000 440	1220	780	450 2000	1225	775
23	80 40	60	20	35 35	35	0
24	40 80	60	20	17 33	25	8
25	320 280	300	20	350 240	295	55
26	4800 3600	4200	600	5300 4000	4650	650
27	1300 1900	1600	300	2500 3000	2750	250
28	440 400	420	20	650 1250	950	300

Note:

	Drop plates	Pour plates
Mean count per sample	*1,482,280*	*1,077,600*
Mean deviation per sample	*264,140*	*119,000*

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SURFACE GROWTH OF BACTERIA ON CELLOPHANE[1]

By G. B. Reed[2] and D. G. McKercher[3]

Abstract

A method is described for the cultivation of bacteria on the surface of sheets of cellophane spread over layers of absorbent material as cotton saturated with any desired fluid medium. The method has proved to be useful in preparing suspensions of bacteria reasonably free from contamination by the culture medium.

In the many undertakings that require the surface growth of bacteria in mass, as the preparation of certain antigens, agar is at times a disturbing factor. During the recent period of agar scarcity a procedure was developed that has more recently been used in order to avoid agar. The procedure consists in spreading a sheet of cellophane over a layer of absorbent material saturated with the desired fluid culture medium and cultivating the organisms on the surface of the cellophane.

The method is of most value where larger surfaces than provided by ordinary Petri dishes are required. Enamel baking dishes, 1 to 2 in. deep, with nearly perpendicular sides, with a slightly larger dish used as a cover, as a Petri dish, are satisfactory. For still larger areas ordinary plastic cafeteria trays* have proved convenient. Where only a small number are used each tray may be conveniently enclosed in a heavy paper envelope with an opening at one end. For larger numbers of trays it is convenient to equip a large rectangular autoclave with racks that will permit sliding one tray above another, without covers, in the manner of a chest of drawers. When the autoclave door is opened the trays may be withdrawn individually or pulled out part way, like drawers, for inoculation. Such an autoclave must be further equipped with a thermoregulator that will permit operation as an autoclave at 120° C. and later as a steam heated incubator.**

Various grades of cellophane, with the exception of "moisture proof" are satisfactory. "Plain cellophane,"*** No. 300, which is listed as 0.00088 in. thick or No. 450, 0.0012 in. thick are satisfactory. These may be obtained in sheets cut to size or in rolls cut to any desired width.

Various absorbent materials have been used to hold fluid media and support the layers of cellophane. Filter pulp, as supplied by Reeve Angel in sheets

about 1 cm. thick, may be cut to fit the trays, enough fluid medium is added to completely saturate the pulp, the cellophane sheet added and firmly rolled down with a photographic print roller. Absorbent cotton, about half the thickness of the ordinary roll, is equally good when similarly treated. Bran, meal, or peat have also been used with good results. Enough fluid medium is added to the material to make a thick paste that is spread roughly in the tray, a sheet of cellophane is added and rolled smooth. It is not necessary to use clarified fluid medium as it filters through the cellophane.

In one large series of cultures the fluid medium consisted of 7% crude corn steep liquor in water without clarification. This was mixed with the following proportions of crushed peat, as used by Lochhead and Thexton (1), for the storage of bacteria, and asbestos fiber as used for insulation.

For 25 trays (15 × 20 in.)

Crude corn steep liquor	1200 ml.
Tap water	16,000 ml.
Crushed peat	2 kgm.
Asbestos fiber	4 kgm.

The acid corn steep and acid peat are neutralized by the alkaline asbestos to give a well buffered medium, after autoclaving, of pH 7.2 to 7.4.

The only difficulty in handling this material is to prevent curling of the cellophane in the autoclave. This can be avoided by preventing the formation of a vacuum as the autoclave cools. A water manometer should be attached to the autoclave. In most autoclaves this can be done very simply by introducing a T in the pipe leading to the pressure gauge and providing the side arm with a valve and a small glass manometer. In operation, as the autoclave cools and the pressure gauge approximates zero, the valve to the manometer may be cracked momentarily at short intervals until the manometer just registers zero. The air port on the autoclave is then opened; this prevents vacuum formation on further cooling. Incidently the procedure is of value in the preparation of coagulated serum or egg slants.

The cellophane surface may be inoculated in a variety of ways. A few drops of inoculum added with a pipette spreads readily with an angle glass rod or the angle rod may be wrapped with gauze and kept moist by adding inoculum drop by drop.

The period of growth and the method of harvesting will vary with the type of organism. Cells of more butyrous growths or the almost woolly growths of some species of *Bacillus*, before sporulation is complete, can be readily harvested by scraping the surface of the cellophane with a square of glass, as a lantern slide cover, and pushing the mass of cells from the edge of the glass into a beaker with a glass spatula. Cell masses in more watery growths, as most *Salmonella* species or spores of *Bacillus* after the vegetative rods have

autolyzed, may be more readily collected with a vacuum device. Such a device may be made of about $\frac{1}{4}$ in. stainless steel tubing in the form of a T with the cross arm 3 to 4 in. long carrying a very narrow slit, 0.2 to 0.5 mm. The long arm of the T, which serves as a handle, is connected by rubber tubing to a receiving vacuum bottle. It is readily possible by this method to collect vegetative cells or spores in the form of a thin paste with the order of 10^{10} to 10^{12} cells per ml.

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METHODS FOR THE DETERMINATION OF THE DISTRIBUTION OF RADIOACTIVE PHOSPHORUS AMONG THE PHOSPHORUS-CONTAINING CONSTITUENTS OF TISSUES[1]

By J. A. McCarter[2] and Ethel L. Steljes[3]

Abstract

The phosphorus-containing constituents of various tissues of the rat were separated into five fractions. These were: inorganic and organic phosphorus soluble in aqueous 10% trichloroacetic acid; phosphorus soluble in 3 : 1 alcohol-ether; phosphorus derived from ribonucleic acid; and phosphorus derived from thymonucleic acid. In order to test the methods, inorganic phosphate, disodium phenyl phosphate, and purified ribonucleic and thymonucleic acids were added to a homogenate of rat liver in 10% trichloroacetic acid. It was possible by separation and analysis of the fractions to obtain a quantitative recovery of the added phosphorus in the appropriate fractions. The extent to which radioactive inorganic phosphate entered the other fractions was measured following its addition to a homogenate of rat liver in 10% trichloroacetic acid. When the fractionation was performed at room temperature it was found that all of the fractions except the organic acid-soluble phosphorus fraction contained negligible amounts of the added radioactive phosphorus. The amount of radioactive phosphorus taken up by this fraction could be reduced to a negligible quantity by working quickly and with cold solutions.

Data are given showing the radioactive and nonradioactive phosphorus contents of the various fractions of liver, spleen, kidney, muscle, thymus gland, lymph node, prostate gland, and testis of the rat 24 hr. after the intraperitoneal injection of a solution of radioactive inorganic phosphate. By analysis of the urine, feces, and remainder of the rat after removal of the above tissues, a quantitative account was made of the injected radioactive phosphate.

Introduction

Other investigators (9, 11) have reported methods for the determination of the distribution of the phosphorus of tissues among the phosphorus-containing constituents but these methods were not well suited to the purposes of the present work. The methods used in this laboratory permit the separation of inorganic and organic phosphorus soluble in 10% trichloroacetic acid, phosphorus soluble in 3 : 1 alcohol–ether, phosphorus derived from ribonucleic acid, and phosphorus derived from thymonucleic acid. The methods used for these separations were based on procedures described by Le Page and Umbreit (6), Delory (2), Bloor (1), and Schmidt and Thannhauser (8). When suitably modified it was possible to combine these procedures to permit the determination of the nonradioactive phosphorus (hereafter referred to as P^{31}) and radioactive phosphorus (P^{32}) contents of each of the above groups of substances present in a small sample of tissue.

The procedure involved the grinding of the tissue in aqueous 10% trichloroacetic acid, boiling the residue with a mixture of 3 : 1 alcohol–ether under

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reflux, treating the extracted residue with 1 N sodium ·hydroxide at 35° C. and acidifying the resulting solution in order to separate ribonucleotide phosphorus and thymonucleotide phosphorus.

The procedures were tested by adding known amounts of phosphorus as inorganic phosphate, disodium phenyl phosphate, purified sheep liver ribonucleic acid, and calf thymus nucleic acid to a homogenate of rat liver in 10% trichloroacetic acid. This was done in order to enrich the corresponding phosphorus fractions of the tissue. Fractionation of the homogenate before and after the addition of the above substances and analysis ·of the fractions resulted in a recovery of over 90% of the phosphorus of each of the added substances in the corresponding fraction.

Before the procedures could be applied to the determination of the distribution of P^{32} among the various fractions it was necessary to determine to what extent exchange of P^{32} took place between them while they were being separated. In order to do this P^{32} as inorganic phosphate was added to a homogenate of normal rat liver in 10% trichloroacetic acid at room temperature and the various fractions were separated and analyzed. It was found that the organic acid-soluble phosphorus fraction contained approximately 10%, the alcohol–ether extract contained 0.004%, the ribonucleotide phosphorus fraction contained 0.004%, and the thymonucleotide phosphorus fraction contained 0.001% of the added P^{32}. When the experiment was repeated with care to work quickly and to keep the trichloroacetic acid solutions cold, the organic acid-soluble phosphorus was found to contain 3.4% of the added P^{32} in one experiment and 2.8% in another. Because it was found necessary to employ cold conditions during part of the fractionation procedure animals were killed by freezing with liquid nitrogen. The tissues were removed and were kept frozen until they were ground in cold trichloroacetic acid.

Tissues of the rat that have been analyzed by the procedures described in this paper are muscle, liver, spleen, kidney, testis, prostate gland, thymus gland, and lymph node. In order to illustrate the application of the methods, the results of a typical experiment on the uptake of P^{32} by the various fractions of the above tissues of the rat are given in detail in this paper.

Experimental

THE DETERMINATION OF NONRADIOACTIVE AND RADIOACTIVE PHOSPHORUS

The present work required the direct determination of the specific activity of a given phosphorus fraction. This was done by preparing a solution of the fraction and determining the P^{31} and P^{32} contents of the solution. Organic material was digested with concentrated nitric and perchloric acids and a portion of the resulting solution was used for the determination of its P^{31} content by King's modification (5) of the method of Fiske and Subbarow (3). Another portion of the same solution was used for the determination of its

P³² content by precipitation of ammonium molybdiphosphate and determination of the β-ray activity of the precipitate. Preliminary experiments showed that after the administration of a small dose of P³² (10 to 20 μc. (microcuries)) to a rat it would not be necessary to use more than 1 mgm. and rarely necessary to use more than 0.2 mgm. of phosphorus derived from tissue in order to obtain a satisfactory measurement of its radioactivity. In order to ensure that the precipitation of ammonium molybdiphosphate would take place under comparable conditions for every sample it was decided to precipitate P³² in association with 1.0 to 1.2 mgm. of phosphorus. It was necessary, therefore, to add nonradioactive phosphorus to every sample that contained less than 1.0 and 1.2 mgm. of P³¹.

The Determination of Nonradioactive Phosphorus
Inorganic Phosphate

Inorganic phosphorus was determined using the method of King (5) except that the volume of solution at the time of color development was 10.0 ml. instead of 15.0 ml. and the time allowed for color development was 30 min. instead of five minutes. These changes were introduced for purposes of convenience. Neither change was found to influence the results of the analysis to a measurable extent.

Total Phosphorus

The method of King was modified further by digestion of organic material with concentrated nitric acid prior to digestion with perchloric acid. This was done in order to decrease the probability of occurrence of explosions. Alcohol–ether extracts of tissue were evaporated to a small volume in the presence of water before the addition of nitric acid.

The phosphorus content of a large mass of organic material such as feces or the carcass of a rat was determined by placing the material in a large beaker and adding enough concentrated nitric acid to cover it. The beaker was covered with a large watch glass and was warmed on an electric hot plate until frothing had subsided and most of the material had dissolved. The temperature was then raised until the nitric acid boiled and more nitric acid was added as necessary until no visible trace of organic material remained. The process of solution of an entire rat required about 8 to 10 hr. for its completion. Finally the solution was transferred with concentrated nitric acid to a suitable volumetric flask and an aliquot part of this solution was used for the determination of its content of P³¹ and P³².

The Determination of Radioactive Phosphorus

Phosphorus was precipitated as ammonium molybdiphosphate by a method based on that described by Willard and Diehl (12). A suitable volume of the solution to be analyzed was measured into a 40 ml. short-cone heavy-duty Pyrex centrifuge tube. Water was added to make the volume 10 ml. and a sufficient volume of a solution of potassium dihydrogen phosphate was added

to make the amount of P^{31} contained by the sample 1.0 to 1.2 mgm. Enough 72% perchloric acid was added so that the mixture contained 1 ml. of the acid. Two milliliters of a 50% saturated solution of ammonium nitrate was added and the mixture was stirred by agitating the tube. The tube was placed in a water bath at 80° C. for 10 to 15 min. and 5 ml. of 5% molybdic acid reagent* were added slowly with agitation. The tube was removed from the bath and allowed to stand at room temperature for one to two hours with occasional mixing. A small proportion of the precipitate was found to float on the surface of the liquid. It was possible to sink this material by adding a small volume of 95% ethanol to the tube in such a way that a layer of alcohol was formed at the top of the liquid. The precipitate was collected by centrifugation and the supernatant liquid was discarded. The precipitate was washed twice by suspending it in 10-ml. portions of 1 : 50 (v./v.) nitric acid and centrifuging. The supernatant liquids were discarded.

The above procedure was tested by weighing the ammonium molybdiphosphate precipitated from solutions containing 1.0 mgm. of P^{31} as phosphate. The average recovery of P^{31} obtained in six such experiments was 99% (standard deviation $= \pm 0.8\%$).

In order to prepare the precipitate for measurement of its radioactivity it was dissolved in a few drops of concentrated ammonium hydroxide and was transferred with water to a small flat-bottomed aluminum dish having a slightly raised edge. The solution was evaporated to dryness under an infrared lamp in such a way that a uniform deposit was obtained over the bottom of the dish.**

In order to determine whether or not a quantitative precipitation of P^{32} could be obtained by the use of the above procedure the following experiment was performed. To a solution of inorganic radioactive phosphate 1 mgm. of P^{31} as phosphate was added and precipitated as ammonium molybdiphosphate. To the supernatant liquid after this precipitation a further 1 mgm. quantity of P^{31} was added. This was also precipitated as ammonium molybdiphosphate in order to carry down any P^{32} left after the first precipitation. The average amount of activity found in the precipitate obtained from the supernatant liquid was equal to 0.32% (standard deviation $= \pm 0.02\%$) of that of the first precipitate. This was taken as evidence for the quantitative nature of the precipitation.

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* *Five per cent molybdic acid reagent was prepared according to the directions of Willard and Diehl (12).*

** *This technique of preparing the precipitate for measurement of its activity has been replaced by the collection of the precipitate on filter paper in a device similar to the Tracerlab E-8 precipitation apparatus (Tracerlab Inc., Boston, Mass.). The precipitate is washed in the apparatus with 1 : 50 (v./v.) nitric acid and with 95% ethanol. It has been found advantageous to complete the washings with a dilute solution of an alcohol-soluble resin in order to prevent the precipitate from falling off the paper when dry. For this purpose we have used a 4% solution of Gelva V-7 (Shawinigan Chemicals Ltd., Montreal) in 95% ethanol. The paper is sucked dry and is mounted in a suitable holder under the window of the counter tube.*

The measurement of the β-ray activity of the precipitate was made using a Geiger-Mueller counter tube of the end-window type and a "scale of 128".* In order to permit the calculation of the activity of test samples relative to a standard and to avoid the necessity of making corrections for decay of P^{32}, the activities of the test samples were compared with those of several identically prepared and mounted samples obtained from aliquot parts of serial dilutions of the parent specimen of the radioactive phosphate solution. Corrections for differences in back scatter and self-absorption in samples containing 1.0 to 1.2 mgm. of P^{31} were not necessary. In order to calibrate the P^{32} samples in absolute units ($\mu c.$) the activities of the standards were compared with that of an identically mounted, weighed quantity of pure U_3O_8 covered with a sheet of aluminum whose thickness was 27 mgm. per sq. cm. The method of calculation of absolute units from this type of measurement has been described by Kamen (4). The activities of all samples were measured with a standard deviation of 2 to 5% of the net counting rate (4).

THE EXTRACTION OF ACID-SOLUBLE PHOSPHORUS

The methods used in the present work for the extraction from tissues of the acid-soluble phosphorus fraction were based on those described by Le Page and Umbreit (6). The following procedure was carried out in a room the temperature of which was 5° to 8° C. The frozen tissue was ground to a powder with a cold stainless steel mortar and pestle. The powdered tissue was transferred while still frozen to a test tube containing 10% trichloroacetic acid. Approximately 5 ml. of 10% trichloroacetic acid were used for small organs such as the prostate gland. Larger volumes (up to 20 ml.) were used when larger organs such as the liver were to be analyzed. The weight of tissue was obtained by weighing the tube and its contents before and after the addition of the frozen powder. The tissue was then ground in the tube by the procedure of Potter and Elvehjem (7) using a pestle made of Plexiglass. The resulting homogenate was transferred to a volumetric flask of suitable size using 10% trichloroacetic acid. The concentration of tissue in the homogenate was such that 10.0 ml. (the volume usually fractionated) contained 0.07 to 0.6 gm. of tissue, depending on the weight of the tissue that was ground. Aliquot parts of the homogenate were taken for the determination of the total P^{31} and total P^{32} contents of the tissues.

Two 10.0 ml. volumes of the homogenate were measured into separate 40 ml. cone-shaped, heavy-wall Pyrex centrifuge tubes and each was treated in the following manner. The tube was centrifuged and the supernatant solution was transferred to a 50 ml. volumetric flask. The residue was washed three times by suspending it in 10–ml. portions of 10% trichloroacetic acid and centrifuging. The supernatant solutions were combined and the volume was made up to 50 ml. with 10% trichloroacetic acid. The residue was set aside for the extraction of phosphorus soluble in alcohol–ether. Duplicate

* *Model 161, Instrument Development Co. Ltd., Chicago, Ill.*

aliquot portions of the trichloroacetic acid extract were taken for the measurement of its content of P^{31} and P^{32}. Other aliquot portions were used for the determination of P^{31} present as inorganic phosphate. The amount of P^{31} present as organic acid-soluble P^{31} was determined by taking the difference between the values obtained for total acid-soluble P^{31} and inorganic acid-soluble P^{31}. Inorganic phosphate was precipitated from the trichloroacetic acid extract by the method of Delory (2) after the addition of 50 μgm. of P^{31} as inorganic phosphate to serve as a "carrier" of radioactive phosphate. The precipitate was dissolved in 1 ml. of 72% perchloric acid and the P^{32} content of the solution was determined. The supernatant and wash liquids were digested with nitric and perchloric acids in order to determine the amount of P^{32} present as organic acid-soluble P^{32}.

It was established by experiment that no appreciable amount of phosphorus was contained in the last of four successive trichloroacetic acid extracts of a sample of tissue. Approximately 0.5 gm. of rat liver was extracted with four 10 ml. portions of 10% trichloroacetic acid and each extract was analyzed separately. The data are recorded in Table I.

TABLE I

THE AMOUNTS OF PHOSPHORUS IN SUCCESSIVE TRICHLOROACETIC
ACID EXTRACTS OF APPROXIMATELY 0.5 GM. OF RAT LIVER

Number of extract	P^{31} present in extract, mgm.	
	Experiment 1	Experiment 2
1	0.415	0.402
2	0.017	0.016
3	0.014	0.016
4	0.006	0.008

The Extraction of Alcohol-Ether-Soluble Phosphorus

The residue obtained after the removal of acid-soluble phosphorus was extracted with ethanol by suspending it and centrifuging it twice with 5–ml. portions of 95% ethanol. The supernatant solutions were removed to a 100 ml. Kjeldahl flask. Ten milliliters of a mixture of ethanol and ether (three volumes of 95% ethanol and one volume of diethyl ether) was than measured into the tube, a Pyrex glass bead was added, a small cold-finger condenser was inserted into the mouth of the tube, and the mixture was boiled under reflux for one-half hour. At the end of this time the tube was centrifuged and the supernatant solution was combined with the ethanol extracts. The extraction with 10 ml. of ethanol–ether was performed three times. After pouring off the fluid from the last extraction the tubes were placed, unstoppered, in a warm water bath until the contents were dry. The contents of the Kjeldahl flask were evaporated to a small volume over

gentle heat, about 5 ml. of water was added, and the contents of the flask were again evaporated to a small volume. Finally, concentrated nitric acid was added and the analysis of the extract was completed as described above.

The above extraction procedure was developed as the result of experiment. Approximately 0.5 gm. of rat liver was extracted with 10% trichloroacetic acid and the residue was extracted with ethanol and with ethanol–ether as described above. Each extract was analyzed separately. The results of the experiment are recorded in Table II.

TABLE II

THE AMOUNTS OF PHOSPHORUS PRESENT IN SUCCESSIVE ETHANOL AND ETHANOL-ETHER EXTRACTS OF APPROXIMATELY 0.5 GM. OF RAT LIVER

Solvent	Number of extract	P^{31} found, mgm.	
		Experiment 1	Experiment 2
Ethanol, 95%	1	0.212	0.278
	2	0.161	0.131
Ethanol–ether, 3 : 1	1	0.089	0.091
	2	0.014	0.012
	3	0.006	0.006
	4	0.002	0.003

In another experiment the period of extraction with boiling ethanol–ether was two hours instead of one-half hour. No more phosphorus was extracted by boiling for the longer time than for the shorter. The results of this experiment are recorded in Table III.

TABLE III

A COMPARISON OF THE AMOUNTS OF PHOSPHORUS EXTRACTED FROM SAMPLES OF RAT TISSUES BY BOILING WITH 3 : 1 ETHANOL– ETHER FOR DIFFERENT PERIODS OF TIME

Tissue	Ethanol-ether-soluble P^{31}, mgm./gm. fresh wt. of tissue	
	One-half hour	Two hours
Liver	1.07	1.08
Thymus	0.54	0.52

THE SEPARATION OF RIBONUCLEOTIDE PHOSPHORUS AND THYMONUCLEOTIDE PHOSPHORUS

For the separation of the ribonucleotide phosphorus and thymonucleotide phosphorus the method of Schmidt and Thannhauser (8) was employed. The method was modified by the use of 1 N sodium hydroxide instead of

1 *N* potassium hydroxide.   This change was introduced in order to avoid the precipitation of potassium perchlorate during the oxidation of the organic material with perchloric acid.

Steudel and Peiser (10) found that ribonucleic acid is quantitatively split into acid-soluble nucleotides when treated with approximately 3% sodium hydroxide at room temperature for 24 hr.   In the present work it was found that 97.5% of the phosphorus of purified calf thymus nucleic acid* was insoluble in 5% trichloroacetic acid after 17 hr. incubation at 35° C. in 1 *N* sodium hydroxide and that 96.5% of the phosphorus of purified sheep liver ribonucleic acid became soluble under the same conditions.

The procedure used for the separation of ribonucleotide phosphorus and thymonucleotide phosphorus was as follows.   To the centrifuge tube containing the dried residue after extraction of the alcohol-ether-soluble phosphorus, 1 *N* sodium hydroxide (10 ml. per gm. fresh weight of tissue) was added. The tube was stoppered and placed in an incubator at 35° C. where it was left overnight.   The next morning the solution was transferred quantitatively to a suitable volumetric flask using 1 *N* sodium hydroxide to wash the tube and to fill the flask to the mark.   Duplicate aliquot portions of the solution were used for the determination of their contents of P^{31} and P^{32}.

To other duplicate aliquot parts of the solution were added 0.2 volume (of the aliquot) of 6 *N* hydrochloric acid and one volume (of the aliquot) of 5% trichloroacetic acid.   The precipitate was separated by centrifugation and washed twice with 5–ml. portions of 2.5% trichloroacetic acid.   The supernatant and wash liquids (containing ribonucleotide phosphorus) were combined and analyzed for their contents of P^{31} and P^{32}.   The precipitate (containing thymonucleotide phosphorus) was also analyzed for its contents of P^{31} and P^{32}.

APPLICATION OF THE FRACTIONATION PROCEDURE

An experiment was performed to test the ability of the above procedures to permit the quantitative separation of phosphorus-containing substances added to a sample of tissue.   The liver of a rat was homogenized in 10% trichloroacetic acid and the homogenate was diluted to a suitable volume. Duplicate aliquot portions of the homogenate were fractionated and the P^{31} contents of the fractions were determined by the procedures described above.

To another aliquot part of the same homogenate were added known amounts of phosphorus in the forms of a solution of potassium dihydrogen phosphate, a solution of disodium phenyl phosphate, a fine suspension of purified sheep liver ribonucleic acid, and ·a solution of purified calf thymus nucleic acid. The mixture was homogenized, made to suitable volume in 10% trichloroacetic acid and duplicate aliquot portions of the homogenate were fractionated and analyzed as described above.   From the results of the analyses of the

* *The authors wish to thank Dr. G. C. Butler for the specimens of purified calf thymus nucleic acid and sheep liver ribonucelic acid used in this investigation.*

fractions of the tissue before and after the addition of the phosphorus-containing substances, the recovery of the phosphorus of the added substances was calculated. The results of this experiment are recorded in Table IV. From

TABLE IV

The Recovery of Known Amounts of Phosphorus Added as Potassium Dihydrogen Phosphate, Disodium Phenyl Phosphate, Purified Sheep Liver Ribonucleic Acid, and Calf Thymus Nucleic Acid to a Homogenate of Rat Liver in Trichloroacetic Acid

Fraction	Amount of P³¹ added, mgm.	Amount of P³¹ recovered, mgm.
Inorganic phosphate P³¹	0.20	0.22
Acid-sol. organic P³¹*	0.28	0.24
Alcohol-ether-sol. P³¹	0.00	0.06
Ribonucleotide P³¹	0.36	0.32
Thymonucleotide P³¹	0.24	0.21

Difference between total acid-soluble P³¹ and inorganic acid-soluble P³¹.

these data it was concluded that it was possible, by application of the procedures described in this communication, to account satisfactorily for the phosphorus of added substances in the appropriate fractions.

The Possibility of Exchange of P³² Between Fractions

Before the procedures described herein could be used to determine the distribution of P³² among the phosphorus-containing constituents of tissues, it was necessary to determine to what extent exchange of P³² took place between the various fractions while they were being separated.

The liver of a rat was homogenized in 10% trichloroacetic acid at room temperature in the presence of a known amount of P³² as inorganic phosphate. As soon as possible after diluting the homogenate to a suitable volume in a volumetric flask duplicate aliquot portions were taken for fractionation and analysis by the above procedures. The homogenate was stored in the refrigerator and other samples were removed for analysis 24 and 48 hr. later. The results of these analyses are recorded in Table V.

The data of Table V show that the P³² added to the homogenate as inorganic phosphate remained almost quantitatively in the acid-soluble phosphorus fraction. About 10% of the P³² was present in the organic acid-soluble phosphorus fraction as a result of exchange of inorganic phosphate with esterified phosphate or as a result of failure to effect a quantitative precipitation of inorganic phosphate by the method of Delory.

It was found possible to decrease the amount of P³² that entered the organic acid-soluble phosphorus fraction by carrying on all operations at a temperature of 5° to 8° C. and by working quickly. When this was done the inorganic

TABLE V

THE EXCHANGE OF P^{32} ADDED AS INORGANIC PHOSPHATE TO A HOMOGENATE
OF RAT LIVER IN TRICHLOROACETIC ACID

The extraction of acid soluble phosphorus was performed at room temperature. The amount
of P^{32} present in a given fraction is expressed in terms of a percentage
of the amount of P^{32} present in the homogenate

Fraction	Time at which fractionation started:		
	Start of expt.	24 hr.	48 hr.
Homogenate	100	100	100
Total acid-sol. P	96.5	93.0	94.7
Inorganic phosphate	85.5	84.7	84.2
Organic acid-sol. P	11.0	8.3	10.5
Alcohol-ether-sol. P	0.002	0.002	0.002
Extracted residue	0.004	0.004	0.005
Ribonucleotide P	0.004	0.003	0.004
Thymonucleotide P	0.001	0.001	0.002

phosphate fraction was found to contain 101.0% and the organic acid-
soluble phosphorus fraction 3.4% of the total P^{32} present in the homogenate.
In a similar experiment the corresponding values obtained were 99% and
2.8% respectively. These data were taken as an indication of the absence
of an appreciable amount of exchange under the conditions of the experiment
and established the quantitative nature of the precipitation of inorganic
phosphate from a trichloroacetic acid extract by the method of Delory.

APPLICATION OF THE PROCEDURE TO EXPERIMENTS WITH THE RAT

In order to illustrate the application of the procedures reported in the
present paper the following typical experiment is described.

A single male hooded rat, three months old and of 279 gm. body weight
was used in this experiment. It received, by intraperitoneal injection from a
calibrated hypodermic syringe and without being anesthetized, approximately
180 μc. of P^{32} as phosphate in a neutral physiological saline solution. The
animal was placed immediately in a cage that permitted the separate collection
of urine and feces. Water and food (Purina Fox Chow Checkers) were
provided ad libitum.

Twenty-four hours after the injection of the solution containing P^{32} the
rat was killed in the following manner. Ten minutes before the expiration
of the 24 hr. experimental period the animal was held by hand in the meta-
bolism cage and was anesthetized by the intraperitoneal injection of a solution
of sodium pentobarbital (50 mgm. per kgm. of body weight). As soon as the
animal became unconscious (usually about five minutes after the injection)
0.10 ml. of a solution of heparin (1000 units per ml., Connaught Laboratories,
Toronto) was injected into one of the external jugular veins and one minute

later 1 ml. of blood was withdrawn from this vein using a different syringe. The animal was then frozen by pouring liquid nitrogen over it. The body of the rat was allowed to soften, but not to thaw in a room of which the tempera-ture was 5° to 8° C. and was placed on a large watch glass for dissection. The liver, spleen, both kidneys, both testes, prostate gland, thymus, muscle from hind legs, and lymph nodes from the neck, axillae, and peritoneal cavity were removed. As soon as the tissues were excised they were placed in tightly stoppered glass weighing bottles and stored in the freezing compart-ment of a refrigerator. The remainder of the rat was placed in a 3 liter beaker. The instruments and watch glass used for the dissection were washed with water that was collected in the beaker. The amounts of P^{31} and P^{32} present in the contents of the beaker were determined.

The feces were collected from the metabolism cage and the cage was washed with several small volumes of water. The washings were combined with the urine and the P^{31} and P^{32} contents of the urine and feces were determined.

Each tissue was frozen with liquid nitrogen, and powdered with a stainless steel mortar and pestle, both of which were kept cold with liquid nitrogen. The ground frozen tissue was homogenized in trichloroacetic acid, was sub-jected to the fractionation procedures described above, and the P^{31} and P^{32} contents of the fractions were determined. The results of this experiment are given in Tables VI and VII.

The data of Table VI show that the sums of the amounts of P^{31} found in the individual fractions of the tissues are always within 15% and usually within 10% of the amounts found in the complete tissue. The sums of the amounts of P^{32} found in the individual fractions were always within 12% and usually within 7% of the amounts of P^{32} found in the complete tissue.

The data of Table VII show that a quantitative recovery of the injected P^{32} was made by analysis of all of the parts of the animal.

Discussion

The methods described in this communication provide a means of deter-mining the distribution of P^{32} among the phosphorus-containing constituents of tissues following the administration of radioactive phosphate to the rat. The phosphorus-containing constituents were separated into groups that may be further fractionated and that are relatively well characterized.

By grinding the tissues in trichloroacetic acid and by fractionating aliquot portions of the resulting homogenate it was possible to obtain accurate sampling of the tissue and good agreement between the results of analyses of corresponding fractions of duplicate aliquots. The use of small amounts of tissues (0.07 to 0.06 gm. fresh weight of tissue) made it possible to perform the fractionation in a single tube thus avoiding losses that otherwise might have occurred through transfer.

TABLE VI

THE DISTRIBUTION OF P31 AND P32 AMONG PHOSPHORUS-CONTAINING FRACTIONS OF TISSUES OF A RAT 24 HR. AFTER THE INTRAPERITONEAL INJECTION OF A SOLUTION CONTAINING APPROXIMATELY 180 μC. OF P32 AS INORGANIC PHOSPHATE

P31 data are expressed as mgm. P31 per gm. fresh weight of tissue. P32 data are expressed as percentage of the dose of P32 administered found per gm. fresh weight of tissue

Fraction	Liver		Kidney		Spleen		Testis		Lymph node		Thymus		Prostate gland		Muscle	
	P31	P32	P31	P32	P31	P32	P31	P32	P31	P32	P31	P32	P31	P32	P31	P32
Complete homogenate	3.28	0.44	2.76	0.32	2.22	0.30	2.18	0.069	3.4	0.34	4.4	0.65	2.25	0.21	2.47	0.15
A. Total acid-sol. P	1.08	0.18	0.79	0.16	0.82	0.15	0.74	0.052	0.71	0.21	1.06	0.21	0.60	0.091	2.2	0.15
Inorganic phosphate	0.51	0.057	0.49	0.069	0.30	0.056	0.52	0.025	0.41	0.069	0.43	0.078	0.34	0.048	0.72	0.063
Organic acid-sol.*	0.57	0.11	0.30	0.047	0.52	0.080	0.22	0.013	0.30	0.050	0.63	0.070	0.26	0.030	1.5	0.065
B. Alcohol-ether-sol. P	1.09	0.22	0.96	0.13	0.44	0.053	0.58	0.012	0.59	0.072	0.56	0.075	0.68	0.083	0.45	0.008
C. Extracted residue	0.9	0.039	0.72	0.033	0.97	0.072	0.69	0.006	2.28	0.095	3.2	0.39	1.3	0.023	0.21	0.002
Ribonucleotide P	0.77	0.045	0.51	0.036	0.57	0.052	0.53	0.006	1.33	0.066	1.81	0.26	0.88	0.038	0.18	0.002
Thymonucleotide P	0.16	—	0.18	—	0.39	0.024	0.11	—	0.95	0.020	1.70	0.16	0.17	—	0.04	—
Sum of A, B, C	3.07	0.44	2.47	0.32	2.23	0.27	2.01	0.070	3.58	0.38	4.8	0.68	2.58	0.20	2.86	0.16

* Organic acid-sol. P31 was measured by subtracting inorganic acid-sol. P31 from total acid-sol. P31.
Organic acid-sol. P32 was measured directly.

TABLE VII

THE DISTRIBUTION OF A KNOWN AMOUNT OF P^{32} IN THE RAT 24 HR. AFTER THE
INTRAPERITONEAL INJECTION OF A SOLUTION OF P^{32} AS PHOSPHATE

The values for the amount of P^{32} found in the tissues are given in terms of
percentage of the dose of P^{32} administered to the rat

Tissue analyzed	Amt. of P^{32} in tissue removed from rat, % of dose P^{32}	Tissue analyzed	Amt. of P^{32} in tissue removed from rat, % of dose P^{32}
Liver	5.69	Bone	2.26
Kidneys	0.72	Blood	0.14
Spleen	0.32	Urine	18.3
Testes	0.22	Feces	22.0
Lymph nodes	0.09	Remainder of rat	52.9
Thymus	0.23		
Prostate gland	0.04	Sum of above	103.3
Muscle	0.38		

By determining P^{31} and P^{32} in the same solution the specific activity of the solution may be measured directly. This measurement is uninfluenced by errors in the weight of the material analyzed and its accuracy depends only on the accuracy with which P^{31} and P^{32} are determined.

The specific activity of the phosphorus of an individual fraction may be influenced by the extent to which this fraction has been prepared free of others and the extent to which exchange of phosphate between the fractions has been eliminated. The data of Table IV provide information concerning the completeness of separation of the fractions from one another. The data of Table V show that little or no exchange of phosphate occurred between inorganic phosphate and alcohol-ether-soluble phosphorus, ribonucleotide phosphorus, and thymonucleotide phosphorus. If the reasonable assumption is made that any exchange of phosphate between these fractions in a trichloroacetic acid medium takes place through the intermediate formation of inorganic phosphate, the data of Table V indicate that the extent of this exchange is negligible.

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ASSESSMENT OF HOSPITAL DIETS[1]

By G. Hunter[2], J. Kastelic[3], and M. Ball[4]

Abstract

Analytical values are given for vitamin A, ascorbic acid, thiamine, riboflavin, niacin, calcium, phosphorus, iron, and protein on 12 diets commonly used in a modern hospital. The foods were analyzed as served to the patient. Assessed on the basis of commonly accepted standards the diets are found to be deficient in various respects, and particularly in the B-vitamins, ascorbic acid, and iron.

Introduction

In view of the valuable findings of the R.C.A.F. nutritional laboratories respecting the diets of air force personnel during the war, at the end of the war, when No. 4 R.C.A.F. Nutritional Laboratory was turned over to the University here, it was thought that an investigation of hospital diets might be of some value in the many-sided problem of convalescence and rehabilitation. Accordingly, with the assurance of the co-operation of an Edmonton hospital, an investigation was undertaken to ascertain the nutritional adequacy of 12 hospital diets, including seven separate Sippy Diets.

All diets were collected at the hospital by a trained dietitian and assayed in the laboratory for vitamin A and carotene, thiamine, riboflavin, niacin, ascorbic acid, calcium, phosphorus, iron, and protein. The Progressive Sippy Diets and the Tube Fed Diet were one day collections. Longer collection periods were not necessary because of the constant and uniform composition of these diets. The Soft and Clear Fluid Diets were collected over a three day period. All other diets were collected over a five day period.

Collection of Samples

At breakfast time when about half the ward trays had been prepared in the hospital diet kitchen one tray was set aside as our sample meal.

From this tray the individual food items were weighed and transferred to a four liter brown screw top bottle—Bottle I—containing 15 ml. glacial acetic acid. The procedure was the same at lunch and at supper. The whole day's food, including between-meal snacks, was thus collected in Bottle I.

For purposes of daily assay additional corresponding samples of vitamin C containing foods were collected over the day in a separate bottle—Bottle II—in a volume of 5% metaphosphoric acid corresponding to the weights of the items.

It should be noted that shells were removed from eggs, stones from stone fruits, the bone from meat, and the skins from oranges or grapefruit before weights were taken and food placed in the bottle. Beets were not collected

because their pigment interferes in colorimetric procedures used in the assay of some of the food constituents. When choices of beverages were permitted at the same meal a compromise was made. If milk was included in the sample meal, then in the next meal only the milk or cream in the tea or coffee was collected.

Bottles I and II were taken at the end of the day to the nutritional laboratory. Appendix I shows the amounts of the food items in the diets.

Preparation of Samples for Analysis

The day's food in Bottle I was mixed in batches in a Waring blendor. Enough water, in measured amount, was added when necessary for mixing. Besides the total weight of the food, the total volume of the mixed day's food was recorded. One-tenth of each day's mixed food was transferred to a brown glass bottle, stored in a refrigerator. The five day aliquots were thoroughly mixed.

All analyses, excepting those for ascorbic acid, were carried out on aliquots from this material.

The contents of Bottle II were homogenized in a Waring blendor, and determinations were carried out on each day's sample. The five days' values were then averaged.

Assay Methods

In general the methods used by the R.C.A.F. were followed.

Thiamine was determined by the fermentation method of Schultz, Atkin, and Frey (15).

Riboflavin was determined by the microbiological method of Snell and Strong (16).

Niacin was determined by the chemical method of Melnick, Oser, and Siegel (11), except in the case of the Sippy Diets where it was determined by the microbiological method described by Snell and Wright (17) and by Kiehl, Strong, and Elvehjem (9).

A comparison of the values for niacin found by the two methods is shown for six diets, as follows:

Diet	Method of assay	
	Microbiological	Chemical
	Niacin, μgm./100 ml. of food suspension	
Low Fat	315	381
Low Salt	264	306
Child Adm. Diabetic	450	420
Full Fluid	210	296
Tube Feeding	880	712
Sippy 1 to 5 days	69	140
Sippy 6th day	66	850
Sippy 7 to 8 days	56	400

The *vitamin A plus carotene* was determined by the Olcott and McCamm (13) modification of the Carr–Price method.

Ascorbic acid was determined by the usual method of titration with 2,6 : dichlorophenolindophenol, on Bottle II collections. Various checks were made for non-vitamin C reductants (18), but the amounts found were insignificant.

Protein—total nitrogen was determined by the Kjeldahl method with a mixture of mercuric oxide (HgO) and powdered selenium (14) with the usual conversion factor 6.25.

As a check on the Sippy Diet results, protein was determined in commercial milks as shown below by the standard method of nitrogen described under (1).

Grade A milk (3.5% fat), summer 1946		Cream—10% fat, summer 1946		Milk from hospital kitchen, winter 1945–46	
Bottle	Protein, %	Bottle	Protein, %	Sample	Protein, %
1	3.13	1	2.76	1	3.21
2	3.18	2	2.75	2	3.18
3	3.09	3	2.78	3	3.23

Calcium was determined by the method of Kramer and Tisdall (10).

Iron was determined by the method of Jackson (8).

Phosphorus was determined by the method of Fiske and Subbarow (4).

Check on Added Vitamins

The Tube Fed Diet was supplemented by certain vitamin preparations indicated in the table below. It is perhaps of some interest to compare the values found by our analytical methods with those calculated for milk, milk powder, and eggs from food tables and those for the vitamin preparations supplied to us by the Division of Pharmacy of the Hospital.

Food items in diet	Vit. A, I.U.	Thiamine, mgm.	Riboflavin, mgm.	Niacin, mgm.	Ascorbic acid, mgm.
Percomorph	10,000				
Betalin		10.00			
B. Plex		1.80	3.55	17.70	
Ascorbic acid					100.0
Milk, 1900 ml.	3200	0.80	3.40	2.10	
Milk powder, 100 gm.	1000	0.34	1.93	1.10	12.0
Eggs, 5	2300	0.30	0.82	0.15	
Total, calculated	16,500	13.24	9.70	21.05	112.0
Total, found	18,200	11.31	9.10	22.88	28.0

It will be thus seen that the only serious discrepancy is in the ascorbic acid values. It appears probable here that the amount of metaphosphoric acid used was insufficient to acidify the mixture and a large amount of the ascorbic acid was oxidized before titration was carried out.

Discussion of Results

All analytical values for the average day's intake are assembled in the chart (Fig. 1). As a guide to dietary energy supply calculated values for calories are included. As a basis for assessment of adequacy of dietary constituents, the Recommended Dietary Allowances of the Food and Nutrition Board, National Research Council, U.S.A. (12) are indicated, as the averages for sedentary men and sedentary women, by hatched bars.

We shall briefly comment* on the diets as illustrated in Fig. 1.

Comments on Diets

Full House.—This is the general diet for the Hospital and its general adequacy should be of some concern to us. Judged on the basis of Recommended Dietary Allowances it is adequate in protein, calcium, phosphorus, iron, and vitamin A, but inadequate in calories, vitamin C, and the B-vitamins, thiamine, riboflavin, and niacin. Its energy value should be increased by about 20%, its vitamin C and riboflavin doubled, its niacin tripled, and its thiamine increased fourfold.

Sippy Diets.—During the first two weeks the diets present a regime of definite insufficiency upon which only a small patient would not lose weight. Throughout the three weeks the diets are adequate only in calcium, phosphorus, and riboflavin. The vitamin A should be doubled and the iron and thiamine should be tripled on the average over the three week period. With respect to ascorbic acid and niacin the diet appears well designed to produce both scurvy and pellagra.

Initial Postulcer.—This diet is adequate in most respects. Its iron should be increased 50% and its niacin at least doubled. It is also barely adequate in thiamine and riboflavin.

Admission Diabetic.—The calorie values would here appear about 25% too low. The iron, riboflavin, and niacin should be increased by 50% and the thiamine increased two to four times. Vitamin C is here adequate.

Low Residue.—The amounts of iron, ascorbic acid, thiamine, and niacin present in this diet should all be about doubled. The diet is in other respects adequate.

Low Salt.—The B-vitamins here should be increased by 50%.

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* *In this connection we should like to note that our comments should not be interpreted in a mechanical fashion. We are aware of the inadequacy of comparing the diets of hospital patients with a very tentative standard for healthy people, but above all we do not want to imply that poor diets should be mechanically supplemented by synthetic vitamins or mineral preparations. The omments are made merely as a guide to the improvement of the whole food diets.*
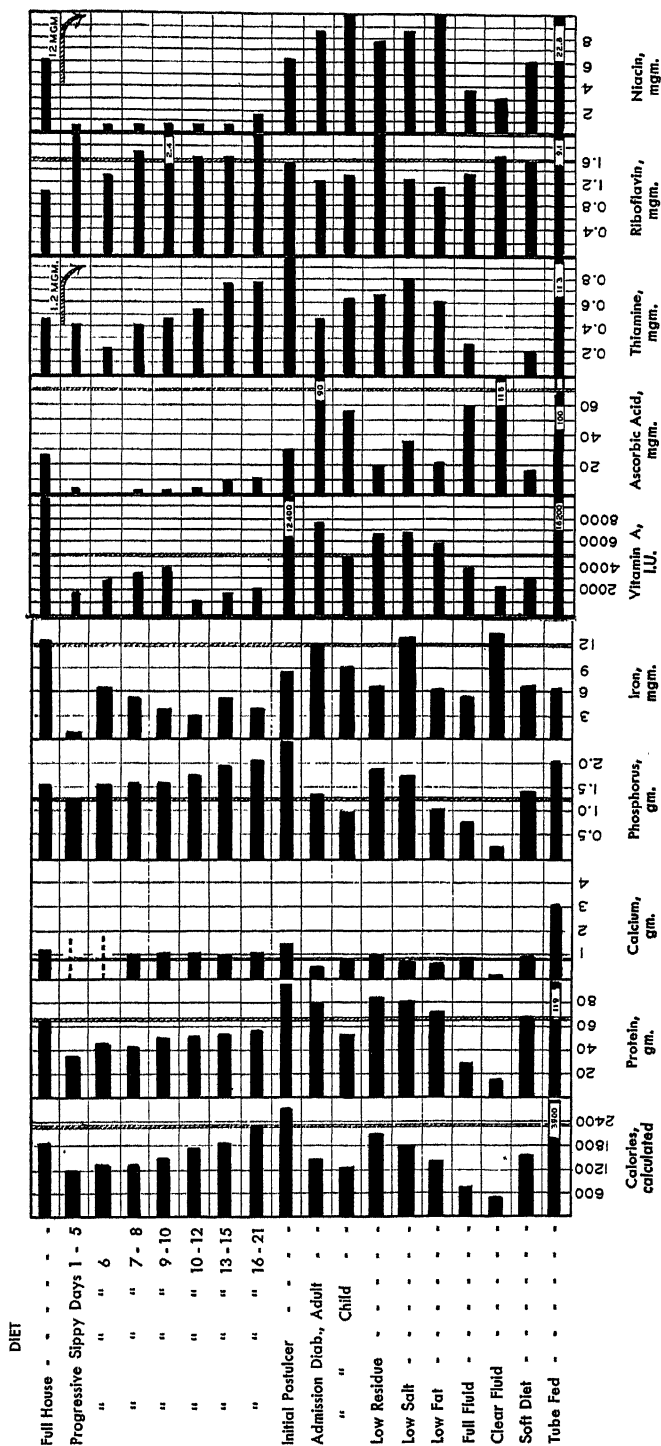
FIG. 1. Hospital diets; daily constituent intake.

Low Fat.—The calorie value of this diet is 50% too low.   Its iron, ascorbic acid, and each of the three B-vitamins should be doubled.

Full Fluid.—These diets are used only over very short periods in acute conditions of the gastrointestinal tract and in association with operative intervention, so one need not be unduly concerned with their low energy values and other deficiencies.

Soft Diet.—This diet has only about 15% of the recommended thiamine allowance.   It is also quite inadequate in iron, ascorbic acid, and niacin.

Tube Feeding.—With its high calorie value, high mineral and vitamin values this diet should quickly repair undernutrition.   It is notable however that the diet contains only half the recommended iron allowance.   The balance of added vitamins is open to question.

Comments on Dietary Constituents

With the exception of only four diets out of 18, the energy value of the diets supplied is appreciably lower than the recommended sedentary allowances.

In general, the protein, calcium, and phosphorus allowances of the patients are met, and sometimes more than met.

Iron is adequate in the Full House Diet, in the Adult Admission Diabetic, in the Low Salt, and in the Clear Fluid Diet.   Iron is deficient in the Sippy Diets, in the Low Fat Diet, in the Soft Diet; and in the Tube Fed Diet.

Except for the Sippy Diet the vitamin A supplied is fairly adequate.

Ascorbic acid is at a satisfactory level in the Admission Diabetic Diets, in the Full Fluid Diet, in the Clear Fluid Diet, and in the Tube Fed Diet.

The B-vitamins, and especially thiamine and niacin, judged on the basis of recommended allowances, are alarmingly low.   Special comment is called for respecting those two constituents.

It should first be noted that the "recommended allowance", with respect to vitamins, is assumed to be at least 50% greater than the minimal requirements for the prevention of definite signs of deficiency.   The diets under study are so low in thiamine and niacin that the question arises as to whether they are meeting minimal requirements for the avoidance of beriberi and pellagra. The question can be answered more clearly for thiamine than for niacin.

Minimal Thiamine Requirements

If we calculate the vitamin/calorie index used by Cowgill (2) in his studies of the incidence of beriberi as observed up to 1934 we find for the daily intake of 0.43 mgm. of thiamine an index: 2276/1900 or 1.15.   Of course our calorie value is an estimate and we do not have an average weight of patient upon which to apply this index precisely, but on Cowgill's standards such an index would just support a patient 40 kgm. or 88 lb. in weight.   It is certain that the average weight of patients in the hospital is considerably above this figure, and as Cowgill found that with an index less than 1.76 there was an

appreciable incidence in the groups studied, so we might expect an appreciable incidence of beriberi in patients after perhaps a month or two on this thiamine intake.

The fact that beriberi does not exist under such circumstances in the hospital is an indication that the Cowgill standards are too high, or the patients' stay in hospital is too short for deficiency signs to appear.

A recent review on *The Thiamin Requirements of Man* by Holt (5) enables us to reach a more definite conclusion on the subject. Holt, on the basis of rigorous criteria, places the minimum requirement for the normal adult "between 0.17 and 0.23 mg. per 1000 calories", for the prevention of definite beriberi. On a mean value of 0.20, and assuming our calorie intake to be about 2000, the thiamine intake of 0.43 just exceeds the minimum. However Holt adds: "A range intake between 0.24 and 0.44 mg. per 1000 calories appears to be protective against thiamin deficiency."

If we take the mean value here of 0.34, then our House Diet should contain at least 0.6 mgm. of thiamine per day.

On the basis of such observations we conclude that the Full House Hospital Diet is at least 50% below conservative thiamine requirements.

Minimal Niacin Requirements

We have no reliable observations on the minimum requirements of niacin to prevent pellagra in humans. It has been calculated (see Dann (3)) that the pellagrous subjects of Goldberger and Wheeler ingested 7.2 mgm. daily. Winters and Leslie (see (3)) in a study of low income groups in Texas found by analysis a mean intake of 4.2 mgm. of niacin per day with no pellagra. Calculations from blacktongue tests on dogs to the 70 kgm. man indicate a requirement of 10 mgm., but this is not regarded as a valid inference as niacin metabolism in man and dog differ.

The Full House Diet containing 5.95 mgm. of niacin, on the basis of present knowledge, could not be regarded as generous. On the three weeks of the Sippy Diet the average daily intake is only 0.93 mgm. But because pellagra is not produced over this period one could not assume that the minimum requirement is less than 0.93 mgm.

The Full House Diet, and several of the others, but especially the Sippy Diets, provide inadequate amounts of niacin.

Results in Relation to Foods Supplied

The prevalence of milk in the diets assures a general adequacy of calcium and phosphorus and adds significantly to the protein and riboflavin. Milk however is low in iron and so there is an average daily intake over the Sippy period of only 4.4 mgm. The main food sources of iron are meats, breads, vegetables, and eggs. There is adequacy of iron in the Full House, Adult

Admission Diabetic, and Low Salt Diets from such sources.   The high iron in the Clear Fluid Diet was mostly obtained from boiled down green vegetable juice.

The high values found in the Full House Diet and the Initial Postulcer Diet for vitamin A are to a large extent accounted for by carrots supplied in two or three meals out of the five day collection.   This emphasizes how readily a poor nutritional picture may be changed to a good one.

Ascorbic acid is adequate only where citrus fruits have been provided. The non-citrus foods used by the hospital are apparently unsuitable to provide an adequate vitamin C intake.   The titrations on the daily vitamin C food collections reflected this fact very strikingly.

The vitamin C should be increased in the Full House Diet, in the Sippy Diet, and in others by increasing the citrus fruits supplied, or by other means, such as preparations from wild rose hips, which are a far richer source of ascorbic acid and abundantly home grown.   Failing natural sources synthetic ascorbic acid should be supplied.

In the Full House Diet it appears that about 20% of both the thiamine and niacin was derived from bread; on an average about 0.1 mgm. thiamine and 2.0 mgm. niacin per day.   From the dietary constituents it can be calculated that there was present in the daily food about 4 oz. of white flour.   Were the white flour replaced by an equal quantity of Canada Approved Flour, the daily diet would gain about 0.33 mgm. of thiamine and 4.1 mgm. niacin (6) thus taking the diet definitely out of the danger zone that exists for those two vitamins.

General

We have chosen the mean allowances of the U.S. National Research Council, for sedentary men and women, as a conservative basis on which to assess particularly the Full House Hospital Diet.   On this basis it has been shown to be low in calories, in all water soluble vitamins, and even dangerously low in thiamine and niacin.

There is no scientific evidence to support the belief that large amounts of vitamins hasten healing and regenerative processes except where there has been previous deficiency.   The average convalescent patient therefore needs no more than the ordinary sedentary individual.   But, as proper nutrition is a fundamental basis of health, and patients are in hospital to regain health, the general adequacy of a hospital diet should not be open to question.   Where there is faulty nutrition in the local population (7) as there is with respect especially to iron and the water soluble vitamins, it would appear that the hospital meals should be designed to counteract such conditions rather than perpetuate and even exacerbate them.

There would seem to be little justification for the continuation of the unsupplemented Sippy regime.   It is definitely established that there is practically no wound healing in scurvy, and vitamin C has a structural function in collagen

formation. There is recent evidence that the daily requirement of vitamin C may be nearer 30 mgm. than 70 mgm. but the Sippy regime provides a daily average of only 4 mgm. The danger of surgical intervention on patients after prolonged periods on the Sippy regime would appear to be considerable.

It would be of little use, even if we were competent, to pursue this discussion into the adequacy of the special diets in meeting the needs for which they are used. The subject of nutritional needs in relation to clinical conditions is very wide and varied, and only the medical practitioner is in a position to judge wisely how his patient should be fed. The dietary analyses and discussion presented in this paper may be helpful to practitioners in making use of nutrition as a means towards the health of their patients.

Finally, it should be emphasized that the diets in the particular hospital here investigated are likely to be at least as good as in the average Canadian hospital.

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Appendix I

House Diet—Full (five day collection)
  Nov. 19 to 23, 1945

Day 1—Nov. 19, 1945

Milk (3½%)	240	gm.
Bread, white	21	"
Bread, rye	20	"
Roll, white	36	"
Butter	13	"
Soda crackers	8	"
Graham crackers	15	"
Cream of wheat	144	"
Egg, scrambled	116	"
Cocoa	167	"
*Baked beans with tomato sauce	128	"
*Cabbage salad	55	"
*Soup, vegetable	138	"
*Parsnips, steamed	92	"
*Potato, steamed	80	"
Roast beef	40	"
Gravy	20	"
Caramel pudding	120	"
*Crab apples, canned	126	"

Total weight = 1579 gm.
Total volume = 2000 ml.

*Analyzed for vitamin C
  Total weight = 574 gm.
  Total volume = 1080 ml.

Day 2—Nov. 20, 1945

Milk (3½%)	395	gm.
Bread, brown	35	"
Bread, white	18	"
Roll, white	45	"
Butter	17	"
Soda crackers	9	"
Graham wafers	15	"
Rolled oats	125	"
Bacon	35	"
Cocoa	156	"
Macaroni and cheese	160	"
*Soup, vegetable	108	"
*Tomatoes, stewed	44	"
*Potato, mashed	96	"
*Carrots, raw, shredded	40	"
Liver, baked	52	"
*Fruit salad (diced fruit)	155	"
Cottage pudding with chocolate sauce	72	"

Total weight = 1577 gm.
Total volume = 2000 ml.

*Analyzed for vitamin C
  Total weight = 420 gm.
  Total volume = 880 ml.

House Diet—Full, cont'd.
  Day 3—Nov. 21, 1945

Milk (3½%)	428	gm.
Bread, brown	19	"
Bread, white	35	"
Roll, white	38	"
Butter	23	"
Soda crackers	9	"
Graham wafers	15	"
*Potato, mashed	85	"
Brex	132	"
Egg, soft cooked	48	"
Cocoa	215	"
*Soup, vegetable	136	"
*Cabbage, steamed	56	"
*Potato, scalloped	85	"
*Lettuce salad	65	"
Meat loaf	81	"
Gravy	52	"
Bologna	56	"
*Prunes, stewed	92	"
Ice cream (Neapolitan)	46	"

Total weight = 1716 gm.
Total volume = 2620 ml.

*Analyzed for vitamin C
  Total weight = 1519 gm.
  Total volume = 1000 ml.

Day 4—Nov. 22, 1945

Milk (3½%)	361	gm.
Bread, brown	15	"
Bread, white	13	"
Roll, white	50	"
Butter	13	"
Soda crackers	9	"
Graham wafers	14	"
Cream of wheat	118	"
Egg, fried	46	"
Egg, devilled	50	"
*Soup, vegetable and macaroni	141	"
*Peas and carrots	73	"
*Cabbage and carrot salad	73	"
*Potato, steamed	87	"
*Potato, baked	100	"
Roast beef	64	"
Vanilla cream	114	"
Sugar cookies	32	"
Cocoa	158	"

Total weight = 1531 gm.
Total volume = 2520 ml.

*Analyzed for vitamin C
  Total weight = 474 gm.
  Total volume = 1000 ml.

Appendix I—*Cont'd.*

House Diet—Full, concl'd.

  Day 5—Nov. 23, 1945

Milk...................	253	gm.
Cocoa..................	200	"
Bread, brown...........	34	"
Bread, white...........	23	"
Roll, white............	40	"
Soda crackers..........	9	"
Graham wafers..........	15	"
Rolled oats............	178	"
Egg, soft cooked, 1, edible portion (E.P)........	47	"
*Soup, barley...	168	"
*Tomatoes, stewed......	130	"
*Potato, mashed........	127	"
*Carrot sticks.........	20	"
Salmon, baked.....	100	"
Shepherd's pie.........	194	"
Jello..................	126	"
Spice cake with icing..	61	"
Butter.................	20	"
(Beets)..	(60)	"

            Total weight = 1745 gm.
            Total volume = 2400 ml.

 *Analyzed for vitamin C
    Total weight = 445 gm.
    Total volume = 900 ml.

Progressive Sippy (1 to 5 days) Oct. 1, 1945

Milk : cream 1 : 4.............	1106	gm.

            Total volume = 1106 ml.

 (Milk 3.5%, cream 10% fat: 1:4)

Progressive Sippy (6th day) Oct. 9, 1945

Milk and cream.	1106	gm.
Eggs, whole, 2, E.P...........	160	"

            Total weight = 1266 gm.
            Total volume = 1150 ml.

Progressive Sippy (7th and 8th days)
   Oct. 22, 1945

Milk and cream...............	1106	gm.
Eggs, whole, 2, E.P...........	116	"
Cereal (cream of wheat)........	85	"

            Total weight = 1307 gm.
            Total volume = 1330 ml.

Progressive Sippy (9th and 10th days)
   Nov. 5, 1945

Milk and cream	1106	gm.
Eggs, whole, 2, E.P...........	130	"
Cereal (cream of wheat)........	170	"
Cocoa......................	85	"

            Total weight = 1491 gm.
            Total volume = 1460 ml.

Progressive Sippy (11th and 12th days)
   Nov. 19, 1945

Milk and cream...............	1106	gm.
Eggs, whole, 2, E.P...........	104	"
Cereal (rolled oats)...........	85	"

Progressive Sippy, concl'd.

Cocoa......................	85	"
Jello (1 serving)..............	110	"
Caramel cream (1 serving).....	115	"

            Total weight = 1605 gm.
            Total volume = 1760 ml.

Progressive Sippy (13th to 15th days)
   Nov. 19, 1945

Milk and cream...............	1106	gm.
Cereal (rolled oats)...........	85	"
Egg, whole, 1, E.P.............	64	"
Custard (1 serving)............	122	"
Milk toast, white bread........	36	"
milk...............	100	"
Jello (1 serving)..............	114	"
Potato, mashed (1 serving).....	130	"
Butter.....................	5	"

            Total weight = 1762 gm.
            Total volume = 1840 ml.

Progressive Sippy (16th to 21st days)
   Nov. 19, 1945

Milk and cream...............	1106	gm.
Cereal (rolled oats)...........	85	"
Toast (white), 2 slices	80	"
Eggs, whole, 2, E.P............	110	"
Ice cream, vanilla (1 serving)...	60	"
Caramel cream (1 serving)......	106	"
Custard (1 serving)............	128	"
Potato, mashed (1 serving).....	90	"
Butter.....................	22	"

            Total weight = 1787 gm.
            Total volume = 1900 ml.

Initial Postulcer Diet (five day collection)
   Nov. 19 to 24, 1945

Day 1—Nov. 19, 1945

Milk (3½%).................	461	gm.
Milk, "Superior", 10%......	599	"
Bread, white................	128	"
Butter.....................	22	"
Eggs, 1, E. P................	54	"
Egg, creamed................	70	"
Cream of wheat..............	150	"
*Jam, peach.................	14	"
Custard....................	56	"
Caramel cream..............	96	"
*Soup, cream of tomato.......	237	"
*Peas, green.................	52	"
*Potato, baked, E.P..........	112	"
*Potato, steamed.............	92	"
Salmon, steamed.............	105	"

            Total weight = 2248 gm.
            Total volume = 2800 ml.

 *Analyzed for vitamin C
    Total weight = 507 gm.
    Total volume = 750 ml.

Appendix I—*Cont'd.*

Initial Postulcer Diet, cont'd.

Day 2—Nov. 20, 1945

Milk (3½%)................	654 gm.
Milk, "Superior", 10%.......	625 "
Bread, white...............	91 "
Butter.....................	18 "
Egg, 1, E.P................	48 "
Rolled oats.................	180 "
*Jam, peach.................	24 "
Applesauce.................	90 "
Cottage pudding with choco-late sauce................	54 "
Cup cake (iced).............	36 "
*Soup, cream of potato.......	182 "
*Tomato juice..............	100 "
*Potato, baked E.P..........	45 "
*Potato, mashed.............	100 "
Cheese, soufflé.............	92 "
Macaroni and cheese........	92 "
*Carrots, steamed...........	35 "

Total weight = 2466 gm.
Total volume = 2920 ml.

*Analyzed for vitamin C

Total weight = 486 gm.
Total volume = 950 ml.

Day 3—Nov. 21, 1945

Milk (3½%)................	650 gm.
Milk, "Superior", 10%......	592 "
Bread, white...............	126 "
Butter.....................	17 "
Egg, 1, E.P................	40 "
Egg, creamed...............	76 "
Cream of wheat.............	112 "
*Jam, peach.................	16 "
*Tomato juice..............	200 "
Ice cream with chocolate sauce	88 "
*Prunes, stewed..............	108 "
Cottage cheese.............	96 "
*Soup, cream of tomato.......	120 "
*Soup, cream of pea..........	130 "
*Potato, scalloped............	118 "
*Potato, mashed.............	76 "
*Peas, green................	48 "

Total weight = 2613 gm.
Total volume = 3080 ml.

*Analyzed for vitamin C

Total weight = 816 gm.
Total volume = 1250 ml.

Initial Postulcer Diet, cont'd.

Day 4—Nov. 22, 1945

Milk (3½%)	523 gm.
Milk, "Superior", 10%......	624 "
Bread, white...............	86 "
Butter.....................	29 "
Eggs, 2, E.P...............	95 "
Cream of wheat............	150 "
*Prunes, stewed..............	70 "
Custard....................	116 "
Vanilla cream..............	122 "
Cottage cheese.............	88 "
*Soup, cream of tomato.......	143 "
*Soup, cream of pea..........	140 "
*Tomato juice..............	106 "
*Potato, baked E.P..........	100 "
*Potato, steamed.............	88 "
*Carrots, steamed...........	52 "

Total weight = 2532 gm.
Total volume = 3315 ml.

*Analyzed for vitamin C

Total weight = 699 gm.
Total volume = 1130 ml.

Day 5—Nov. 23, 1945

Milk (3½%)................	600 gm.
Milk, "Superior", 10%.......	574 "
Bread, white...............	101 "
Butter.....................	23 "
Egg, 1, E.P................	36 "
Rolled oats.................	143 "
*Tomato juice..............	93 "
*Jam, peach.................	13 "
Jello......................	156 "
*Fruit juice, mixed..........	110 "
Custard....................	100 "
Cottage cheese.............	68 "
*Soup, cream of potato.......	112 "
*Soup, cream of carrot........	153 "
*Potato, mashed.............	106 "
*Potato, steamed.............	80 "
Salmon, steamed...........	66 "
(Beets)	(74) "

Total weight = 2534 gm.
Total volume = 3000 ml.

*Analyzed for vitamin C

Total weight = 667 gm.
Total volume = 1200 ml.

Appendix I—*Cont'd.*

Admission Diabetic Diet (five day collection)
  Jan. 15 to 20, 1946

Day 1—Jan. 15, 1946

Milk (3½%)................	240 gm.
Bread, brown..............	60 "
Butter....................	30 "
Rolled oats...............	180 "
Egg, soft cooked, 1, E.P.....	55 "
Broth.....................	160 "
Beef, cold, sliced	60 "
Beef, roast.................	60 "
*Potato, baked..............	50 "
*Potato, steamed............	90 "
*Beans, yellow..............	100 "
*Peas, green................	50 "
*Plums, greengage, canned....	150 "
*Peaches, canned............	150 "
*Grapefruit.................	150 "

Total weight = 1585 gm.
Total volume = 2520 ml.

*Analyzed for vitamin C
  Total weight = 740 gm.
  Total volume = 1250 ml.

Day 2—Jan. 16, 1946

Milk (3½%)................	240 gm.
Bread, brown..............	60 "
Butter....................	30 "
Brex......................	180 "
Egg, soft cooked, 1, E.P......	55 "
Broth.....................	180 "
Beef, roast.................	60 "
Cheese, Canadian...........	40 "
Macaroni..................	60 "
*Potato, steamed.....	90 "
*Tomatoes, stewed..........	40 "
*Turnips, steamed......... ..	75 "
*Applesauce................	150 "
*Pears, canned..............	150 "
*Cherries, Royal Anne, canned	150 "

Total weight = 1560 gm.
Total volume = 2540 ml.

*Analyzed for vitamin C
  Total weight = 655 gm.
  Total volume = 1100 ml.

Admission Diabetic Diet, cont'd.

Day 3—Jan. 17, 1946

Milk (3½%)................	240 gm.
Bread, brown..............	60 "
Butter....................	40 "
Cream of wheat............	180 "
Egg, soft cooked, 1, E.P......	45 "
Broth.....................	190 "
Beef, cold, sliced...........	90 "
Beef, ground...............	60 "
*Potato, steamed...........	140 "
*Tomatoes, stewed..........	60 "
Beets, steamed.............	75 "
*Lettuce...................	40 "
*Apricots, canned...........	150 "
*Fruit salad (cherries, peaches)	150 "
*Prunes, stewed..	50 "
Roll, white................	30 "
*Oranges, 2, E.P............	150 "

Total weight = 1750 gm.
Total volume = 3100 ml.

*Analyzed for vitamin C
  Total weight = 740 gm.
  Total volume = 1350 ml.

Day 4—Jan. 18, 1946

Milk (3½%)................	240 gm.
Bread, brown..............	90 "
Butter....................	60 "
Rolled oats................	180 "
Egg, soft cooked, 1, E.P......	55 "
Broth.....................	200 "
Salmon, steamed	60 "
Cottage cheese.............	60 "
Cheese, Canadian..........	20 "
*Potato, steamed............	90 "
*Potato, baked..............	50 "
*Peas, green, canned.........	65 "
*Lettuce...................	20 "
*Carrots, grated.............	30 "
*Oranges, E.P.....	225 "
*Pears, canned..............	150 "
*Plums, greengage, canned....	150 "

Total weight = 1745 gm.
Total volume = 2360 ml.

*Analyzed for vitamin C
  Total weight = 780 gm.
  Total volume = 1320 ml.

Appendix I—*Cont'd.*

Admission Diabetic Diet, concl'd.

Day 5—Jan. 19, 1946

·Milk (3½%)................	240 gm.
Bread, brown..............	60 "
· Roll, white.................	40 "
Butter.....................	50 "
Cream of wheat............	180 "
Egg, soft cooked, 1, E.P.....	45 "
, Egg, hard cooked, 1, E.P....	30 "
Broth.....................	220 "
, Chicken, cold, sliced.........	60 "
, Veal, roast.................	60 "
*Potato, steamed.............	90 "
*Potato, baked..............	50 "
*Beans, green, canned........	75 "
*Turnip, steamed.............	75 "
*Peas, green, canned.........	35 "
*Applesauce.................	150 "
*Plums, greengage, canned....	150 "
*Peaches, canned.............	150 "
*Oranges, E.P...............	220 "

Total weight = 1980 gm.
Total volume = 3000 ml.

*Analyzed for vitamin C
Total weight = 995 gm.
Total volume = 1350 ml.

Child's Admission Diabetic Diet (five day collection) Feb. 18 to 22, 1946

Day 1, Feb. 18, 1946

Milk (3½%)	540 gm.
Bread, brown..............	45 "
Butter.....................	30 "
Soda crackers, 1.............	5 "
Cream of wheat............	90 "
Broth.....................	110 "
Beef, roast.................	30 "
Egg, soft cooked, 1, E.P.....	45 "
Egg, poached...............	55 "
*Potato, steamed.............	60 "
*Spinach....................	100 "
*Beans, green...............	100 "
*Apricots, canned...........	100 "
*Applesauce.................	100 "
*Apple, 1, raw..............	100 "
*Grapefruit, half.............	105 "

Total weight = 1615 gm.
Total volume = 2660 ml.

*Analyzed for vitamin C
Total weight = 665 gm.
Total volume = 1190 ml.

Child's Admission Diabetic Diet, Cont'd.

Day 2—Feb. 19, 1946

Milk (3½%)................	540 gm.
Bread, brown..............	45 "
Butter.....................	30 "
Soda crackers, 1.............	5 "
Brex......................	90 "
Broth.....................	215 "
Beef, cold, sliced...........	30 "
Egg, soft cooked, 1, E.P......	45 "
Egg, poached...............	60 "
*Potato, steamed.............	60 "
*Beans, green...............	100 "
*Peas, green.................	35 "
*Tomato juice...............	100 "
*Cherries, Royal Anne, canned	100 "
*Apricots, canned...........	100 "
*Apple, 1, raw..............	200 "

Total weight = 1755 gm.
Total volume = 2450 ml.

*Analyzed for vitamin C
Total weight = 695 gm.
Total volume = 1200 ml.

Day 3, Feb. 20, 1946

Milk......................	540 gm.
Bread, brown..............	45 "
Butter.....................	30 "
Soda crackers, 1.............	5 "
Rolled oats.................	90 "
Broth.....................	205 "
Beef, roast.................	30 "
Cheese, Canadian...........	20 "
Egg, soft cooked, 1, E.P......	45 "
*Potato, steamed.............	60 "
*Peas, green.................	35 "
*Beans, green...............	30 "
*Celery, raw.................	20 "
*Lettuce....................	30 "
*Tomato slices...............	20 "
*Tomato juice...............	100 "
*Orange, 1..................	75 "
*Peaches, canned.............	100 "
*Pears, canned..............	100 "

Total weight = 1580 gm.
Total volume = 2000 ml.

*Analyzed for vitamin C
Total weight = 570 gm.
Total volume = 1050 ml.

Appendix I—*Cont'd.*

Child's Admission Diabetic Diet, cont'd.

Day 4, Feb. 21, 1946

Milk (3½%)................	540	gm.
Bread, brown..............	45	"
Butter....................	35	"
Soda crackers, 1............	5	"
Cream of wheat............	90	"
Broth....................	205	"
Lamb, cold, sliced..........	30	"
Cottage cheese.............	30	"
Egg, soft cooked, 1, E.P.....	55	"
*Potato, steamed............	60	"
*Carrots...................	50	"
*Beans, wax.................	50	"
*Turnip sticks...............	30	"
*Tomato slices..............	50	"
*Celery, raw................	20	"
*Apple juice................	100	"
*Orange, 1..................	80	"
*Plums, greengage...........	100	"
*Pears, canned..............	100	"

Total weight = 1675 gm.
Total volume = 2000 ml.

*Analyzed for vitamin C
Total weight = 640 gm.
Total volume = 1100 ml.

Day 5, Feb. 22, 1946

Milk (3½%)................	540	gm.
Bread, brown..............	45	"
Butter....................	40	"
Soda crackers, 1............	5	"
Cream of wheat............	90	"
Broth....................	235	"
Salmon, steamed...........	30	"
Cottage cheese.............	30	"
Egg, soft cooked, 1..........	55	"
*Potato, steamed............	60	"
*Peas......................	35	"
*Tomatoes, canned...........	100	"
*Applesauce.................	100	"
*Orange, 1..................	80	"
*Peaches, canned............	100	"
*Apple juice................	100	"

Total weight = 1645 gm.
Total volume = 1500 ml.

*Analyzed for vitamin C
Total weight = 575 gm.
Total volume = 1000 ml.

Low Residue Diet—(five day collection)
Jan. 15 to 19, 1946

Day 1—Jan. 15, 1946

Milk (3½%)................	560	gm.
Bread, white................	100	"
Butter....................	30	"
Milk, "Superior", 10%.......	115	"
Egg, soft cooked, 1, E.P......	50	"
Egg, creamed...............	90	"
*Soup, cream of tomato.......	170	"
Salmon loaf.................	60	"
*Potato, mashed.............	135	"
*Potato, baked..............	80	"
Rolled oats.................	125	"
*Plum juice.................	175	"
*Jam, peach.................	10	"
Custard....................	95	"
Ice cream, vanilla...........	55	"

Total weight = 1850 gm.
Total volume = 2560 ml.

*Analyzed for vitamin C
Total weight = 570 gm.
Total volume = 1000 ml.

Day 2—Jan. 16, 1946

Milk (3½%)................	495	gm.
Bread, white................	135	"
Butter....................	30	"
Milk, "Superior", 10%......	60	"
Cream of wheat............	130	"
Egg, soft cooked, 1, E.P......	50	"
*Soup, cream of carrot.......	125	"
Soup, tomato and beef.......	100	"
Cheese, creamed.............	95	"
Macaroni and cheese.........	200	"
*Potato, mashed.............	80	"
*Potato, creamed.............	65	"
*Tomato juice...............	185	"
*Jam, peach.................	10	"
*Applesauce, purée...........	80	"
Custard....................	160	"

Total weight = 2000 gm.
Total volume = 2820 ml.

*Analyzed for vitamin C
Total weight = 545 gm.
Total volume = 1050 ml.

Appendix I—*Cont'd.*

Low Residue Diet, cont'd.

Day 3—Jan. 17, 1946

Milk (3½%)................	480 gm.
Bread, white................	70 "
Butter.....................	30 "
Milk, "Superior", 10%......	65 "
Cream of wheat.............	150 "
Egg, soft cooked, 1, E.P......	55 "
Egg, creamed...............	80 "
*Soup, cream of tomato.......	200 "
Chicken, cold, sliced.........	35 "
*Potato, mashed.............	190 "
*Tomato juice...............	50 "
*Jam, peach.................	15 "
Custard....................	95 "
Cottage pudding............	120 "
Roll, white.................	40 "

Total weight = 1675 gm.
Total volume = 2510 ml.

*Analyzed for vitamin C
Total weight = 455 gm.
Total volume = 1000 ml.

Day 4—Jan. 18, 1946

Milk (3½%)................	520 gm.
Bread, white................	90 "
Butter.....................	30 "
Milk, "Superior", 10%......	50 "
Rolled oats.................	165 "
Egg, soft cooked, 1, E.P......	50 "
Soup, cream of pea and beet..	90 "
Soup, cream of carrot (strained).................	110 "
Cottage, cheese.............	95 "
Salmon, steamed............	75 "
*Potato, mashed.............	100 "
*Potato, scalloped............	120 "
*Tomato juice...............	95 "
*Orange juice...............	90 "
*Jello, lemon................	75 "
Custard....................	100 "
*Jam, peach.................	15 "

Total weight = 1870 gm.
Total volume = 2200 ml.

*Analyzed for vitamin C
Total weight = 495 gm.
Total volume = 1050 ml.

Low Residue Diet, concl'd.

Day 5—Jan. 19, 1946

Milk (3½%)................	365 gm.
Bread, white................	70 "
Butter.....................	30 "
Milk, "Superior", 10%......	100 "
Cream of wheat.............	150 "
Egg, soft cooked, 1, E.P......	45 "
*Soup, cream of pea purée....	240 "
Egg, creamed...............	150 "
Salmon, creamed............	50 "
*Potato, mashed.............	70 "
*Potato, baked...............	90 "
*Tomato juice...............	240 "
Chicken, cold, sliced.........	45 "
Custard....................	110 "
*Pudding, lemon.............	115 "
*Jam, peach.................	15 "
Roll, white.................	40 "

Total weight = 1925 gm.
Total volume = 2820 ml.

*Analyzed for vitamin C
Total weight = 770 gm.
Total volume = 1350 ml.

Low Salt Diet—(five day collection)
Jan. 28 to Feb. 1, 1946

Day 1—Jan. 28, 1946

Milk (3½%)................	50 gm.
Milk, "Superior", 10%......	190 "
Bread, white, salt-free (s.f.)..	80 "
Butter, s.f..................	25 "
Cream of wheat, s.f.........	170 "
Egg, soft cooked, 1, E.P......	55 "
Soup, cream of beet........	85 "
Chicken, cold, sliced........	50 "
Beef, roast.................	40 "
*Potato, steamed............	90 "
*Potato, baked...............	100 "
*Peas, green.................	65 "
*Spinach....................	55 "
*Peaches, canned............	55 "
Custard....................	120 "
*Jam, peach.................	10 "
*Grapefruit, half............	95 "

Total weight = 1335 gm.
Total volume = 2700 ml.

*Analyzed for vitamin C
Total weight = 470 gm.
Total volume = 1000 ml.

Appendix I—*Cont'd.*

Low Salt Diet, cont'd.

Day 2—Jan. 29, 1946

Milk (3½%)................	155	gm.
Milk, "Superior", 10%......	65	"
Bread, white, s.f............	95	"
Butter, s.f.................	30	"
Cream of wheat, s.f.........	160	"
Egg, soft cooked, 1, E.P.....	50	"
*Soup, cream of tomato.......	130	"
Beef, roast.................	60	"
Beef, cold, sliced...........	45	"
*Potato, steamed.............	95	"
*Potato, baked...............	30	"
*Carrots....................	60	"
*Spinach....................	60	"
*Orange, half................	35	"
*Plums, canned..............	110	"
*Jam, peach.................	15	"

Total weight = 1285 gm.
Total volume = 2780 ml.

*Analyzed for vitamin C
Total weight = 535 gm.
Total volume = 1030 ml.

Day 3—Jan. 30, 1946

Milk, "Superior", 10%.......	205	gm.
Bread, white, s.f............	115	"
Butter, s.f.................	30	"
Cream of wheat, s.f.........	170	"
Egg, soft cooked, 1, E.P.....	55	"
*Soup, cream of tomato.......	100	"
Chicken, cold, sliced........	55	"
Turkey, cold, sliced.........	35	"
*Potato, steamed.............	145	"
*Potato, baked...............	75	"
Beets, diced................	45	"
*Peas, green.................	50	"
*Cranberry sauce.............	20	"
*Tomato juice...............	75	"
*Jam, peach.................	20	"
Cake, chocolate with icing....	110	"
Cake, apple upside down.....	185	"

Total weight = 1490 gm.
Total volume = 3080 ml.

*Analyzed for vitamin C
Total weight = 485 gm.
Total volume = 960 ml.

Low Salt Diet, cont'd.

Day 4, Jan. 31, 1946

Milk, "Superior", 10%.......	165	gm.
Bread, white, s.f............	95	"
Butter, s.f.................	25	"
Cream of wheat, s.f.........	200	"
Egg, soft cooked, 1, E.P.....	65	"
*Soup, cream of green bean...	90	"
Beef stew, plain.............	85	"
Beef, cold, sliced...........	65	"
*Potato, steamed.............	215	"
*Parsnips...................	50	
*Tomatoes, stewed, canned....	125	
*Orange, half................	30	"
Plums, canned..............	125	"
Pudding, bread.............	115	"

Total weight = 1450 gm.
Total volume = 2920 ml.

*Analyzed for vitamin C
Total weight = 510 gm.
Total volume = 950 ml.

Day 5—Feb. 1, 1946

Milk, "Superior", 10%......	215	gm.
Bread, white, s.f............	135	"
Butter, s.f.................	30	"
Brex......................	150	"
Egg, soft cooked, 1, E.P.....	55	"
Egg, poached..............	45	"
*Soup, cream of potato......	90	"
Salmon, steamed...........	90	"
*Potato, steamed.............	110	"
*Tomatoes, stewed, canned....	125	"
*Peas, green.................	60	"
*Orange, half................	40	"
Custard...................	75	"
Ice cream.................	50	"

Total weight = 1270 gm.
Total volume = 2370 ml.

*Analyzed for vitamin C
Total weight = 425 gm.
Total volume = 950 ml.

Appendix—*Cont'd.*

Low Fat Diet—(*five day collection*)
Jan. 28 to Feb. 1, 1946

Day 1—Jan. 28, 1946

Milk (3½%)................	260 gm.
Bread, white................	70 "
Bread, brown...............	10 "
Butter.....................	5 "
Cream of wheat.............	200 "
Chicken, cold, sliced........	40 "
Beef, roast.................	60 "
*Potato, steamed............	120 "
*Potato, baked..............	75 "
*Spinach, canned............	80 "
*Peas, green................	45 "
*Tomato juice...............	150 "
*Cherries, canned...........	90 "
*Peaches, canned............	65 "
*Fruit juice, mixed..........	90 "
*Jam, peach.................	40 "

Total weight = 1400 gm.
Total volume = 2620 ml.

*Analyzed for vitamin C
  Total weight = 755 gm.
  Total volume = 1200 ml.

Day 2—Jan. 29, 1946

Milk (3½%)................	220 gm.
Bread, white................	95 "
Butter.....................	10 "
Cream of wheat.............	165 "
Beef, roast.................	60 "
Beef, cold, sliced...........	55 "
*Potato, steamed............	90 "
*Potato, baked..............	40 "
*Carrots...................	60 "
*Spinach...................	70 "
*Tomato juice...............	150 "
*Soup, tomato...............	100 "
*Peaches, canned............	90 "
*Plums, canned..............	90 "
*Jam, peach.................	50 "

Total weight = 1345 gm.
Total volume = 2790 ml.

*Analyzed for vitamin C
  Total weight = 740 gm.
  Total volume = 1250 ml.

Low Fat Diet, cont'd.

Day 3—Jan. 30, 1946

Milk (3½%)................	275 gm.
Bread, white................	75 "
Cornflakes..................	25 "
Turkey, cold, sliced.........	40 "
Chicken, cold, sliced........	45 "
*Potato, steamed............	120 "
*Potato, baked..............	50 "
*Peas, green................	45 "
Beets, diced................	40 "
*Tomato juice...............	75 "
*Soup, tomato...............	100 "
*Prune whip................	80 "
*Peaches, canned............	95 "
*Jam, peach.................	50 "

Total weight = 1115 gm.
Total volume = 2300 ml.

*Analyzed for vitamin C
  Total weight = 615 gm.
  Total volume = 1100 ml.

Day 4—Jan. 31, 1946

Milk (3½%)................	250 gm.
Bread, white................	80 "
Butter.....................	5 "
Cream of wheat.............	190 "
Stew, beef, plain............	100 "
Beef, cold, sliced...........	70 "
*Potato, steamed............	220 "
*Parsnips..................	60 "
*Carrots...................	55 "
*Peach juice................	50 "
*Soup, green bean...........	90 "
*Cherries, canned...........	85 "
Pudding, cornstarch, vanilla..	85 "
*Jam, peach.................	45 "

Total weight = 1385 gm.
Total volume = 2380 ml.

*Analyzed for vitamin C
  Total weight = 605 gm.
  Total volume = 1050 ml.

Appendix—*Cont'd.*

Low Fat Diet, concl'd.

Day 5—Feb. 1, 1946

Milk (3½%)................	405 gm.
Bread, white................	115 "
Butter.....................	5 "
Cream of wheat.............	150 "
Salmon, steamed...........	90 "
Chicken, cold, sliced........	45 "
*Potato, steamed.............	90 "
*Potato, baked..............	35 "
*Peas, green.................	60 "
Beets, diced................	30 "
*Tomato juice...............	105 "
*Soup, potato...............	100 "
*Peaches, canned............	110 "
*Cherries, canned...........	85 "
*Jam, peach.................	60 "

Total weight = 1485 gm.
Total volume = 2700 ml.

*Analyzed for vitamin C
  Total weight = 645 gm.
  Total volume = 1120 ml.

Full Fluid Diet (five day collection)
  Feb. 18 to 22, 1946

Day 1—Feb. 18, 1946

Milk 3½%..................	640 gm.
Gruel, cream of wheat.......	50 "
*Soup, cream of pea..........	120 "
*Soup, cream of carrot and pea	
(strained)................	135 "
*Grapefruit juice.............	145 "
*Tomato juice...............	150 "
*Peach juice.................	120 "

Total weight = 1360 gm.
Total volume = 1460 ml.

*Analyzed for vitamin C
  Total weight = 670 gm.
  Total volume = 1120 ml.

Day 2—Feb. 19, 1946

Milk (3½%)................	620 gm.
Gruel, rolled oats............	10 "
*Soup, cream of carrot	
(strained)................	135 "
*Soup, cream of potato	
(strained)................	110 "
*Tomato juice...............	390 "
Jello.......................	155 "
Ice cream..................	50 "

Total weight = 1470 gm.
Total volume = 1900 ml.

*Analyzed for vitamin C
  Total weight = 635 gm.
  Total volume = 1150 ml.

Full Fluid Diet, cont'd.

Day 3—Feb. 20, 1946

Milk (3½%)................	615 gm.
Gruel, rolled oats...........	40 "
*Soup, cream of corn (strained)	125 "
*Soup, cream of tomato	
(strained)................	115 "
*Tomato juice...............	225 "
*Apple juice.................	120 "
Jello.......................	120 "

Total weight = 1360 gm.
Total volume = 1460 ml.

*Analyzed for vitamin C
  Total weight = 585 gm.
  Total volume = 1200 ml.

Day 4—Feb. 21, 1946

Milk (3½%)	560 gm.
Gruel, cream of wheat.......	90 "
*Soup, cream of potato	
(strained)................	130 "
*Soup, cream of onion	
(strained)................	115 "
*Tomato juice...............	120 "
*Apple juice.................	125 "
*Grapefruit juice.............	100 "
Jello.......................	110 "

Total weight = 1350 gm.
Total volume = 1700 ml.

*Analyzed for vitamin C
  Total weight = 590 gm.
  Total volume = 1100 ml.

Day 5—Feb. 22, 1946

Milk (3½%)................	360 gm.
Gruel, cream of wheat.......	50 "
*Soup, cream of tomato	
(strained)................	105 "
*Soup, cream of carrot	
(strained)................	115 "
*Tomato juice...............	120 "
*Peach juice.................	130 "
*Grapefruit juice.............	100 "

Total weight = 980 gm.
Total volume = 1000 ml.

*Analyzed for vitamin C
  Total weight = 570 gm.
  Total volume = 900 ml.

Appendix I—*Concl'd.*

Clear Fluid Diet (3 day collection)
Mar. 5 to 7, 1946

Day 1—Mar. 5, 1946

Broth......................	215 gm.
Jello......................	275 "
*Tomato juice..............	135 "
*Apple juice................	155 "
*Orange juice (fresh).......	120 "
Total weight =	900 gm.
Total volume =	870 ml.

*Analyzed for vitamin C
  Total weight = 410 gm.
  Total volume = 750 ml.

Day 2—Mar. 6, 1946

Broth......................	245 gm.
Jello......................	230 "
*Tomato juice..............	265 "
*Apple juice................	170 "
Total weight =	910 gm.
Total volume =	900 ml.

*Analyzed for vitamin C
  Total weight =   435 gm.
  Total volume = 1120 ml.

Day 3—Mar. 7, 1946

Broth......................	215 gm.
Jello......................	135 "
*Apple juice................	150 "
*Tomato juice..............	130 "
*Grapefruit juice (fresh)......	140 "
Total weight =	770 gm.
Total volume =	920 ml.

*Analyzed for vitamin C
  Total weight = 420 gm.
  Total volume = 930 ml.

Soft Diet (three day collection)
Mar. 5 to 7, 1946

Day 1—Mar. 5, 1946

Milk (3½%)................	100 gm.
Roll, white.................	40 "
Butter......................	10 "
Cream of wheat.............	120 "
Egg, soft cooked, 1, E.P......	65 "
Cheese soufflé..............	115 "
Macaroni and cheese.........	175 "
*Potato, mashed.............	90 "
*Tomato juice...............	120 "
*Soup, vegetable (strained)....	160 "
*Soup, creamed onion (strained)...............	120 "
*Applesauce.................	125 "
Custard....................	135 "
Total weight =	1375 gm.
Total volume =	2000 ml.

*Analyzed for vitamin C
  Total weight =   615 gm.
  Total volume = 1100 ml.

Soft Diet, cont'd.

Day 2—Mar. 6, 1946

Milk (3½%)................	295 gm.
Roll, white.................	40 "
Bread, white................	60 "
Butter......................	15 "
Soda crackers...............	20 "
Rolled oats.................	150 "
Egg, soft cooked, 1, E.P......	55 "
Egg, scrambled..............	100 "
Cottage cheese..............	130 "
*Soup, vegetable (strained)....	120 "
*Soup, cream of corn (strained)	125 "
*Potato, mashed.............	105 "
*Tomato juice...............	130 "
*Peaches, canned............	90 "
Chocolate blanc·mange.......	120 "
Total weight =	1555 gm.
Total volume =	2100 ml.

*Analyzed for vitamin C
  Total weight = 570 gm.
  Total volume = 1000 ml.

Day 3—Mar. 7, 1946

Milk (3½%)................	295 gm.
Roll, white.................	50 "
Butter......................	25 "
Bread, white................	30 "
Soda crackers...............	15 "
Brex.......................	145 "
Egg, soft cooked, 1, E.P......	60 "
Egg, creamed...............	155 "
*Soup, cream of vegetable (strained)...............	155 "
*Soup, cream of celery (strained)................	110 "
*Potato, mashed.............	100 "
*Potato, baked..............	110 "
*Tomato juice...............	150 "
*Banana....................	80 "
Custard....................	110 "
Total weight =	1590 gm.
Total volume =	2000 ml.

*Analyzed for vitamin C
  Total weight = 705 gm.
  Total volume = 1150 ml.

Tube Feeding, Date of collection, 1 feeding,
Feb. 23, 1946

Milk, "Superior", 10%..1077.3 gm. (38 oz.)	
Milk (3½%)...........907.2 gm. (32 oz.)	
Sugar.......................	1½ cups
Eggs........................	5
Skim milk powder............	1 cup
Vitamin C tablet.............	1
B-plex (4 drams).............	4 tsp.
Betalin tablet (1 cc.) (10 mgm.).	1
Percomorph..................	8 drops
Total volume =	2600 ml.

ASSESSMENT OF DIETS: ANALYSIS VERSUS COMPUTATION FROM FOOD TABLES[1]

By G. Hunter,[2] J. Kastelic,[3] and M. Ball[4]

Abstract

Analytical values for nine constituents of 12 diets are compared with corresponding values computed from food tables on the same diets. Factors tending to concordance or divergence in the values obtained by the two methods are discussed.

Introduction

The assessment of diets by direct analysis immediately before their ingestion is indisputably the best means of determining their nutritive value. Assuming accuracy in the collection of the diets, and also reliability of the analytical techniques used for measuring the food constituents—which assumptions are practicably approachable—the direct analytical method can be regarded as capable of yielding a high degree of correctness. Its limitation lies in yielding information valid only for a short period. This is partially overcome by collecting diets over a period of about a week, as has been done in the preceding paper. This week's information might be regarded as approximately valid for a month, or roughly valid for a season; many factors enter such judgments. Perhaps the most important consideration that tends to be overlooked is that an institutional or family diet that proved to be good or deficient during one week of the year is likely to be similar, under similar circumstances—especially economic—at any other time of the year.

On the other hand the assessment of diets by computation from food tables must inevitably carry uncertainties because of the great variability in the composition of natural foods and the greater variability brought about by modern methods of food processing. No amount of analysis and no statistical treatment of the results can overcome the uncertainty of choice of a basic value to compute a constituent of any particular diet.

However, despite the theoretical shortcomings of the best computation methods the fact remains that they have a large measure of usefulness. There is no question that competent dietitians can readily assess diets approximately for most constituents of interest in nutrition today. The extensive literature on food analysis, summarized in food tables, makes this possible. The effects of seasonal variation, storage, cooking, and various other factors can be

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approximated by experienced computers, and practically the whole of the literature on dietary surveys is dependent on this method—applied with greatly varying amounts of observational data.

There has been, however, a perceptible tendency to approach the more direct analytical method. A recent paper by Kaser *et al.* (7) compares "calculated and determined calorie and vitamin contents of mixed diets". The investigators here collected three-day dietary intake records from a population of about 1100 people of all ages. "From these records 80 representative diets for 1 day were selected for duplication, 40 each from the fall and spring periods". The diets were all prepared and cooked under the supervision of the investigators.

Largely because of the rather elaborate organization required for direct analysis there are few recorded comparisons between analytical and computed values. Thus when planning the study reported in the previous paper it was decided to collect the data necessary for subsequent computation of values from food tables, as a test of the computation method.

Methods

A list was made of the weight of every food item for each day for all the diets listed in the appendix to the preceding paper.*

A list was then made of all food items with columns of food table values of the items studied, and containing under each food item the amount present each day in each diet.

From this list the total protein, calcium, thiamine, etc. were summed for each diet over the period studied. All sums were divided by the number of days the diets were collected, so that all results are finally expressed on an average daily basis.

References 1–5 and 9–12 show the food tables used.

In the use of the food tables we endeavored to choose the basic value for the computing of each constituent of each food item as that most likely to apply to our conditions. As far as possible allowance was made for cooking, seasonal variation in food composition, and like factors.

In a few instances, where probable values were not obtainable from the literature, we used our own analytical values, and a few data from unpublished tables used in a previous nutritional survey (6).

Results

In Table I are assembled the values as found by analysis alongside the corresponding values found by computation from food tables.

There are appended to Table I calculated values for carbohydrate, fat, and calories in the diets, with sedentary values for men and women as recommended by the Food and Nutrition Board, National Research Council, U.S.A. (8).

* *Hunter, G., Kastelic, J., and Ball, M. Assessment of hospital diets. Can. J. Research, E, 26 : 347–366. 1948.*

TABLE I

HOSPITAL DIETS: DAILY CONSTITUENT INTAKE. VALUES OBTAINED BY ANALYSIS COMPARED WITH VALUES COMPUTED FROM FOOD TABLES

Diet	Vit. A + carotene, I.U. Analysis	Computed	Ascorbic, mgm. Analysis	Computed	Thiamine, mgm. Analysis	Computed	Riboflavin, mgm. Analysis	Computed	Niacin, mgm. Analysis	Computed	Calcium, gm. Analysis	Computed	Phosphorus, gm. Analysis	Computed	Iron, gm. Analysis	Computed	Protein, gm. Analysis	Computed	CHO, gm. Computed	Fat, gm. Computed	Cal- ories Computed
Full House	9700	9187	25	54	0.43	1.04	1.08	1.96	5.95	9.72	1.29	0.89	1.66	1.49	12.46	13.12	80.3	75.6	239.6	74.0	1900
Progressive Sippy																					
1 to 5 days	1700	3910	3	2	0.40	0.36	2.06	1.55	0.76	0.95	—	1.06	1.24	0.91	1.76	2.21	35.5	34.7	46.5	96.1	1200
6th day	2700	5495	—	2	0.21	0.58	1.38	2.14	0.76	1.05	—	1.14	1.68	1.25	6.12	6.53	45.4	55.2	47.6	114.5	1400
7 to 8 days	3200	5060	1	2	0.40	0.52	1.72	1.98	0.75	1.02	1.04	1.13	1.60	1.17	5.45	6.05	43.2	53.9	77.1	109.4	1400
9 to 10 days	3700	5340	2	2	0.44	0.58	2.48	2.12	0.82	1.12	1.05	1.23	1.64	1.31	4.67	7.39	53.0	62.6	116.3	114.7	1500
11 to 12 days	1000	5145	4	2	0.53	0.68	1.68	2.15	0.82	1.42	1.06	1.27	1.76	1.32	3.17	5.91	53.1	60.3	117.5	117.0	1700
13 to 15 days	1600	4815	9	15	0.74	0.74	1.66	2.03	1.83	2.50	0.96	1.19	1.91	1.23	5.15	5.97	55.7	58.4	153.5	114.0	1900
16 to 21 days	2000	6060	11	11	0.76	0.93	2.09	2.53	6.78	3.51	1.14	1.34	2.09	1.45	4.37	7.98	58.1	70.9	180.9	144.8	2300
Initial Postulcer	12,400	8074	29	60	0.97	1.27	1.57	3.07	8.17	6.41	1.57	1.61	2.63	1.88	8.77	11.50	100.7	97.6	259.4	129.5	2700
Admission Diabetic																					
(Adult's)	7600	6132	90	110	0.44	0.99	1.22	1.44	9.65	11.75	0.49	0.77	1.35	1.40	11.90	13.15	80.8	74.1	217.7	70.7	1500
(Child's)	4800	8579	56	86	0.62	0.85	1.29	1.81	7.23	6.08	0.89	1.10	1.00	1.28	9.12	10.24	56.9	52.0	166.4	67.1	1300
Low Residue	6400	5254	20	69	0.65	1.00	2.01	2.16	8.75	6.27	1.02	1.11	1.88	1.37	7.23	10.05	86.8	80.8	221.0	83.5	2100
Low Salt	6600	6844	34	61	0.80	0.82	1.25	1.22	9.45	10.15	0.71	0.58	1.20	0.97	13.30	11.93	82.3	70.0	228.4	68.0	1800
Low Fat	5800	7578	21	65	0.61	0.76	1.10	1.16	3.72	10.64	0.68	0.69	1.06	0.93	6.69	11.35	74.2	62.9	229.2	28.5	1400
Full Fluid	3800	1796	58	87	0.24	0.55	1.31	1.39	3.76	0.72	0.89	0.73	0.77	0.63	5.56	3.25	30.7	27.6	97.8	23.0	800
Clear Fluid	2200	632	118	70	—	0.30	1.70	0.35	5.05	0.25	0.11	0.18	0.20	0.21	13.45	2.00	17.6	10.3	90.8	6.1	400
Soft Diet	3000	3712	15	47	0.20	0.95	1.55	1.87	2.90	3.71	0.96	0.74	1.46	1.17	7.12	10.34	70.3	67.1	173.2	69.9	1600
Tube Feeding	18,200	16,500	2	120	11.31	11.24	9.10	9.70	22.88	21.05	3.12	3.73	2.11	3.21	6.50	8.90	119.3	130.8	464.0	170.0	3900
Men, sedentary		5000	75		1.2	1.2	1.6		12		0.8	0.8			12.0		70				2500
Women, sedentary		5000	70		1.2	1.2	1.5		12		0.8	0.8			12.0		60				2100

Discussion

On comparing analytical and computed values for the different constituents a greater measure of concordance is found than might have been expected. Adequacy or deficiency is in general made plain by either value. Both methods show clearly that in nearly all diets there is more than adequacy of calcium and phosphorus and less than adequacy of iron, ascorbic acid, and thiamine. Such general concordance is evidence for the general trustworthiness of the computation method based on accurate food amounts.

The values for iron by the two methods are surprisingly concordant with the exception of that of the Clear Fluid diet. The juice of green vegetables which escaped our computation method was responsible for the relatively high iron value found by analysis.

The greatest divergence between analytical and computed values is found in the vitamins. The vitamin A by analysis in the Sippy diets is rarely half, and in one case only one-fifth of the computed values. It appears that the computation factors chosen for milk (170 I.U. per 100 ml.), for cream (400 I.U. per 100 ml.), and perhaps also that for eggs, were too high for these Edmonton foods for the month of October.

It is of interest to note that in the more varied diets the analytical and computed values for vitamin A are fairly concordant: and most likely not because our factors for other items were more accurate but because in a varied diet errors in the choice of basic computing factors have a tendency to cancel each other. As a corollary to this we see that in the Sippy diet an error in choice of computing factor throws the values all out in one direction; e.g. computed vitamin A values are all too high, computed phosphorus all too low—but in the varied diets the divergence for the one constituent is likely to swing in either direction—too high or too low. By induction we might state it as a general principle that the greater the number of different food items in a diet the greater the chances that the computed constituent values will be correct.

It is obvious that such a rule will not apply to an unstable constituent like ascorbic acid if analysis is not carried out promptly or adequate means adopted to prevent oxidation before analysis. In such a case the computing factor used is likely to be so erroneously high that constituent values for all diets will analyze consistently low.

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